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IN VITRO AND IN VIVO RADIOLIGAND BINDING STUDIES FOR THE N-METHYL-D-ASPARTATE RECEPTOR AND THE 5-HYDROXYTRYPTAMINE TRANSPORTER

by

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Submitted in accordance with the requirements for the degree

of

Doctor of Philosophy

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Department of Pharmacology

1996

To my parents, Chiemi and my daughters

In accordance with the requirements of the University of Edinburgh, I declare that this thesis has been composed by myself, and the work presented herein is my own.

Kiyoharu Shirakawa

Abstract

The work presented in this thesis was carried out in the Department of Pharmacology, University of Edinburgh from 1989 to 1992. This thesis contains *in vitro* and *in vivo* radioligand binding studies for the N-methyl-D-aspartate (NMDA) receptor, the 5-HT_{1A} receptor and the 5-HT transporter.

In chapter 2, a novel NMDA antagonist FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride; (+)FR), which was developed in New Drug Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan, was characterised using [^{3}H]MK-801 binding to rat brain membranes. As observed with (+)MK-801, the affinity of (+)FR was increased when 10 μM L-glutamate was added in the assay buffer. (+)FR inhibited [^{3}H]MK-801 binding to rat cortical synaptosomal membranes in the presence of added L-glutamate (10 μM) with a Ki value of 40 nM although (+)FR was 12-fold less potent than (+)MK-801. These results indicate that (+)FR is non-competitive NMDA antagonist. [^{3}H](+)FR synthesised by tritiation of (+)Cl-FR (mono-chlorinated aromatic precursor of (+)FR) was successfully purified by an open column method. *In vivo* distribution of intravenous injection of [^{3}H](+)FR in rats was analysed. Intravenous injection of [^{3}H](+)FR resulted in a rapid accumulation of radioactivity in rat brain.

In chapter 3, the possibility of using derivatives of citalopram (selective 5-HT uptake inhibitor) as a photoaffinity or SPET ligand for the 5-HT transporter was evaluated using 5-azido-citalopram (5-AC) (photoaffinity ligand) and 5-iodo-citalopram (5-IC) (SPET ligand).

5-AC had high affinity (Ki = 1.65 nM) for [^{3}H]citalopram binding sites being only 1.8-fold less potent than citalopram itself. In the presence of 5-AC, repeated U.V. irradiation (15 W) of rat cortical membranes produced a significant 20% reduction respectively in the Bmax value for [^{3}H]citalopram binding compared with control, indicating that 5-AC covalently bound to the [^{3}H]citalopram binding site. Therefore radiolabelled 5-AC may provide a tool for isolation and characterisation of the 5-HT transporter.

5-IC was 5.8-fold less potent than citalopram as an inhibitor of [^{3}H]5-HT uptake into rat cortical synaptosomes with a Ki value of 11.5 nM. This compound had high affinity (Ki = 4.0 nM and 2.9 nM) for [^{3}H]paroxetine and [^{3}H]citalopram binding sites in rat cortical membranes respectively, being only 1.9- and 3.1-fold less potent than citalopram itself. The pharmacological profile of radio-iodinated 5-IC ([^{125}I]5-IC) binding in rat cortical membranes was consistent with results from [^{3}H]citalopram binding experiments. Intravenous injection of [^{125}I]5-IC resulted in a rapid accumulation of radioactivity in rat brain. At 2hr after injection, highest levels of specific [^{125}I]5-IC binding was observed in the brainstem and midbrain which are known to be rich in serotonergic neuronal cell bodies. Pretreatment with paroxetine caused a significant reduction in specific [^{125}I]5-IC binding in the midbrain, rostral cortex and caudal cortex. These results may indicate that [^{125}I]5-IC is a potential SPET ligand for the 5-HT transporter in human brain.

In chapter 4, the cellular localisation of 5-HT$_{1A}$ binding sites and 5-HT uptake sites in the rat frontal cortex and hippocampus were examined using three different neurotoxins,

5,7-DHT, ibotenate and AMPA. Serotonergic lesions by 5,7-DHT produced a reduction in binding site density for [^{3}H]citalopram binding in both the cortical and hippocampal membranes. The lesions of basal forebrain cholinergic neurones using either ibotenate or AMPA did not effect the binding affinity (K_D value) and density (Bmax value) for [^{3}H]8-OH-DPAT and [^{3}H]citalopram binding in either cortical or hippocampal membranes. In conclusion, firstly, the 5-HT transporter sites are located on terminals of serotonergic neurons in the cortex and hippocampus, but not on cholinergic terminals in the cortex. Secondly, the 5-HT_{1A} receptors are not localised on cholinergic terminals in the cortex, or serotonergic terminals in the cortex and hippocampus.

List of publications arising from this thesis

Abstracts

Hodgkiss J.P., Sherriffs H.J., Cottrell D.A., Shirakawa K., Kelly J.S., Kuno A., Ohkubo M., Butcher S.P. and Olverman H.J. (1993), FR115427 is a non-competitive NMDA receptor antagonist in rat brain, Int. Union. Physiol. Sci. Abstr., 44.

Lawrence J.A., Shirakawa K., Olverman H.J., Kelly J.S. and Butcher S.P. (1991), Effects of ibotenate, AMPA & 5,7-DHT lesions on 5-HT transporter sites in rat cortex and hippocampus, Soc. Neurosci. Abstr., 17, 467.18.

Refereed Abstracts

Lawrence J.A., Shirakawa K., Olverman H.J., Kelly J.S. and Butcher S.P. (1991), The localisation of 5-HT$_{1A}$ and 5-HT uptake binding sites in the rat forebrain, Br. J. Pharmacol., 102, 241P.

Lawrence J.A., Shirakawa K., Olverman H.J., Kelly J.S. and Butcher S.P. (1991), Comparison of AMPA and ibotenic acid lesions in rat cortex, Br. J. Pharmacol., 104, 258P.

Full Papers

Hodgkiss J.P., Sherriffs H.J., Cottrell D.A., Shirakawa K., Kelly J.S., Kuno A., Ohkubo M., Butcher S.P. and Olverman H.J. (1993), Neurochemical and electrophysiological studies on FR115427, a novel non-competitive NMDA receptor antagonist, Eur. J. Pharmacol., 240, 219-227.

Lawrence J.A., Olverman H.J., Shirakawa K., Kelly J.S. and Butcher S.P. (1993), Binding of 5-HT$_{1A}$ receptor and 5-HT transporter ligands in rat cortex and hippocampus following cholinergic and serotonergic lesions, Brain Res., 612, 326-329.

Sherriffs H.J., Shirakawa K., Kelly J.S., Olverman H.J., Kuno A., Okubo M. and Butcher S.P. (1993), Characterisation of the binding of [^{3}H]FR115427, a novel non-competitive NMDA receptor antagonist, to rat brain membranes, Eur. J. Pharmacol., 247, 319-324.

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Contents

Abbreviations

The abbreviations used in this thesis are in according with the guidelines set out in the Pharmacological Reviews.

5-AC	: 5-azido-citalopram
ACh	: acetylcholine
AD	: Alzheimer's disease
AMPA	: DL-α-amino-3-hydroxy- 5-methylisoxazole-4-propionic acid
L-AP3	: L-2-amino-3-phosphonopropionic acid
L-AP4	: L-2-amino-4-phosphonobutanoic acid
D-AP5	: D-2-amino-5-phosphonopentanoic acid
AP7	: 2-amino-7-phosphonoheptanoic acid
AUC	: area under the radioactivity concentration-time curve from time zero to infinity
BIMU8	: (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-iso-propyl-2-oxo-1H-benzimidazol-1-carboxamide hydrochloride
BRL43694	: (endo-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-1-methyl-imidazole-3-carboxamide; granisetron
L-CCG-I	: 2S,1'S,2'S-2-(2'-carboxycyclopropyl)glycine
CGP37849	: DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid
CGP39551	: carboxyethylester of CGP37849
CGP39653	: D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid
CGS19755	: cis-4-phosphonomethyl-2-piperidine carboxylic acid
ChAT	: choline acetyltransferase
(+)Cl-FR	: mono-chlorinated aromatic precursor of (+)FR
7-Cl-KA	: 7-chloro-kynurenic acid
CNQX	: 6-cyano-7-nitroquinoxaline-2,3-dione
CNS	: central nervous system
S-4CPG	: S-4-carboxyphenylglycine
CPP	: 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid
D-CPPene	: D-(E)-3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid
CSF	: cerebrospinal fluid
DA	: dopamine

DAU6285	: endo-6-methoxy-8-methyl-8-azabicyclo[3.2.1] oct-3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1 -carboxylate hydrochloride
DCG-IV	: (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine
%D/g	: % injected dose per g wet weight tissue
5,7-DHT	: 5,7-dihydroxytryptamine
%D/ml	: % injected dose per ml
DNQX	: 6,7-dinitro-quinoxaline-2,3-dione
FMHW	: full width half maximum
FMM	: fluoro-methyl-MK-801
(+)FR	: (+)-1-methyl-1-phenyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride; FR115427
(-)FR	: (-)-1-methyl-1-phenyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride
(±)FR	: (±)-1-methyl-1-phenyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride
GDW	: glass distilled water
GR113808	: [1-[2-[(methylsulphonyl-amino)ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate
GR127935	: N-(4-methoxy-3-(4-methyl-1-piperazinyl)phenyl)-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) (1,1-biphenyl)-4-carboxamide
GR38032	: (±)-1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl]-^{4}H-carbazol-4-one; ondansetron
GR43175	: 3-(2-dimethylamino)ethyl-N-methyl-1H-indole-5-methane sulfonamide; sumatriptan
GR65630	: 3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone
(+)HA-966	: (+)-3-Amino-1-hydroxypyrrolid-2-one
5-HT	: 5-hydroxytryptamine
5-IC	: 5-iodo-citalopram
ICS205-930	: (3-α-tropanyl)1H-indole-3-carboxylic acid ester; tropisetron
L-689,560	: 4-trans-2-carboxy-5,7-dichloro-4-phenylamino-carbonylamino-1,2,3,4-tetrahydroquinoline
L-701,324	: 7-chloro-4-hydroxy-3-[3-(phenoxy)phenyl] quinolin-2-(1H)-one

L-705,022	: 7-chloro-4-hydroxy-3-[3-(3-thienyloxy)phenyl] quinolin-2-(1H)-one
LSD	: lysergic acid diethylamide
LTP	: long-term potentiation
MCA	: middle cerebral artery
MCPG	: (RS)-α-methyl-4-carboxyphenylglycine
mCPP	: 1-(3-chlorophenyl) piperazine
MDL72222	: 1-αH-3α-5αH-tropan-3-yl-3,5-dichlorobenzoate
MDMA	: 3,4-methylenedioxy-methamphetamine
mGluRs	: metabotropic glutamate receptors
MK-801	: (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine maleate; dizocilpine
NA	: noradrenaline
nbM	: nucleus basalis of Meynert
NBQX	: 6-nitro-7-sulphamoylbenzo(f)quinoxaline-2,3-dione
NEM	: N-ethylmaleimide
NMDA	: N-methyl-D-aspartic acid
NR1	: NMDA receptor subunit NMDAR1
NR2	: NMDA receptor subunit NMDAR2
8-OH-DPAT	: 8-hydroxy-2-(di-n-propylamino)-tetralin
PCA	: p-chloroamphetamine
PCP	: N-(1-phenylcyclo-hexyl)piperidine; phencyclidine
PCPA	: p-chlorophenylalanine
PET	: positron emission tomography
r	: correlation coefficient
SB200646A	: N-(1-methyl-5-indolyl)-N'-(3-pyridyl)urea hydrochloride
SDZ205557	: 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino)ethyl ester
SL820715	: (±)-α-(4-chlorophenyl)-4-[(4-fluorophenyl)methyl]-1-piperidineethanol
SPET	: single photon emission tomography
SSRI's	: selective 5-HT uptake inhibitors
$t_{1/2}$	: half-life
TCP	: N-(1-thienyl)-cyclohexyl-3,4-piperidine
TFA	: trifluoroacetic acid
TLC	: thin layer chromatography
trans-ACPD	: trans-1-amino-1,3-cyclopentanedicarboxylic acid
UV-5-AC	: U.V. irradiated 5-AC

WAY-100135: N-tert-butyl-3-4(2-methoxy-phenyl-piperazin-1-yl-2-phenylpropanamide dihydrochloride

WAY-100635: N-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridyl)cyclohexane-carboxamide trihydrochloride

YM90K : 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione hydrochloride

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The molecular sites of drug action, i.e. those protein molecules with which drugs must interact to produce their pharmacological effects, are called specific receptors. The development of selective, high affinity radioligands are critical for progress in the area of receptor pharmacology, since structure-activity relationship studies with such compounds are based on the interaction between the drug molecule and the receptor binding site and can provide important information for future design of new drugs. These radiolabelled ligands which interact with a specific binding site are used as tools for the study and characterisation of numerous neurotransmitter receptors. During the past 15 years, *in vitro* radioligand binding assays with brain membrane preparations have become an important technique for studying receptors in the brain. However membrane binding assays are not suitable for analysis of the detailed anatomical distribution of neurotransmitter receptors. For this purpose, Young and Kuhar (1979) originally developed *in vitro* receptor autoradiography which involves incubation of slide-mounted microtome tissue sections with receptor specific radioligands. Autoradiography has contributed to the characterisation and localisation of receptors in the central nervous system (CNS).

1.1.1 PET and SPET Ligands

The advent of positron emission tomography (PET) and the development of suitable receptor- or site-specific agents has made it possible to study neurotransmitter systems in living human brain. Suitable positron emitting radionuclides are

mainly carbon-11 ($t_{1/2}$ = 20 min) and fluorine-18 ($t_{1/2}$ = 110 min). For example, the dopaminergic system can be visualised both pre- and postsynaptically using 6-[^{18}F]fluoro-L-DOPA (Garnett et al., 1983) and 3-N-[^{11}C]methyl spiperone (Wong et al., 1984), respectively. Single photon emission tomography (SPET) is an alternative neuroimaging technique for studying neurotransmitter receptors of the human brain. In this case, radioligands labelled with Iodine-123 ($t_{1/2}$ = 13 hr) are suitable for SPET studies. For example, [^{123}I]3-quinuclidinyl-4-iodobenzilate has been used to obtain images of muscarinic acetylcholine receptor distribution in human brain including that of a patient with Alzheimer's disease (Eckelman et al., 1984; Holman et al., 1985). SPET imaging studies in man using [^{123}I]iodobenzamide, a dopamine D_2 receptor antagonist, have shown specific regional uptake in the basal ganglia, an area known from <u>in vitro</u> studies to have a high density of dopamine D_2 receptors (Kung et al., 1990). Although it is well recognised that the resolution of SPET (full width half maximum; FWHM = 7-15 mm) is lower than that of PET (FWHM = 4-7 mm) (Phelps and Maziotta, 1985), it has a number of advantages. Because of their longer half-lives, SPET can be carried out with commercially available radioisotopes whereas for PET an on site cyclotron is required and the equipment is more complex and difficult to maintain. Moreover, again related to the half-life of the radioisotopes, multiple images can be obtained over a longer time period following a single dose of radioligand with SPET allowing more complex studies to be undertaken. Thus in the longer term SPET may be more suitable for a widespread clinical application.

1.1.2 Photoaffinity Ligands

Photoaffinity labelling is a powerful technique for biochemical investigation of receptors. Photoaffinity ligands which are capable of forming a covalent bond at or near the binding site after photoactivation with light, are useful for isolation and cloning of native receptors. The radiolabelled photoaffinity ligand-receptor protein complex can be isolated and characterised by electrophoresis after all noncovalently bound ligand is removed by an appropriate procedure such as extensive dialysis, gel filtration and gradient ultracentrifugation. From this material it is then often possible to obtain amino acid sequence information which can be used to design oligonucleotides suitable for screening of an appropriate cDNA library. In general, unlike photoaffinity ligands, reversibly-bound ligands even of very high affinity will dissociate during chromatographic, electrophoretic or other procedures which could be used to isolate receptor protein. Therefore, the ability to label the ligand binding site covalently with a radiolabelled photoaffinity ligand has allowed significant progress to be made toward characterisation of receptors. For example, the nicotinic acetylcholine receptor α-subunit was isolated and identified from *Torpedo marmorata* by the photoaffinity ligand [^{3}H]-p-(dimethylamino)-benzenediazonium fluoroborate (Dennis et al., 1988). Using [^{3}H]flunitrazepam as a photoaffinity ligand, the benzodiazepine receptor protein was identified and visualised by electron microscopic autoradiography in rat brain tissue (Möhler et al., 1980). Generally, photoaffinity ligands contain diazo or azido groups, both of which lose nitrogen by photoactivation. These intermediates, carbenes and nitrenes

from diazo and azido groups respectively, are extremely unstable and react with the ligand binding site (Hanstein, 1979; Fedan et al, 1984; Cavalla and Neff, 1985). These properties are quite suitable for photoaffinity labelling studies. However most diazo compounds are light sensitive and hydrolyzed or attacked by nucleophiles such as Cl^- in acidic solution (Bayley and Knowles, 1977). In addition some diazo compounds are subject to photochemical Wolff rearrangements through ketene intermediates (Bayley and Knowles, 1977). Thus chemical instability and susceptibility to internal rearrangements has made carbene precursors of limited use. The nitrenes show a great deal of diversity in their reactions and some of their reactions produce a ligand binding site covalent bond (Fig.1.1) (Cavalla and Neff, 1985). Among azido compounds, arylazides are most often chosen as photoaffinity ligands because they are chemically stable in the absence of light and are less susceptible to rearrangements than other nitrene precursors (Bayley and Knowles, 1977; Hanstein, 1979). Therefore a number of radiolabelled arylazides have been developed as photoaffinity ligands. For example, 4-amino-6,7-dimethoxy-2-[4-[5-(4-azido-3-[^{125}I]iodophenyl)pentanoyl]-1-piperazinyl] quinazoline (α_1 adrenergic receptor) (Leeb-Lundberg et al., 1984), [^{3}H]3-methyl-6-chloro-9-azido-1H-2,3,4,5-tetrahydro-3-benzazepine (α_2 adrenergic receptor) (Regan et al., 1986), p-azido-m-[^{125}I]iodobenzylcarazolol (β adrenergic receptor) (Lavin et al., 1982), [^{3}H]-p-azidoatropine methyl iodide (muscarinic acetylcholine receptor) (Cremo and Schimerlik,

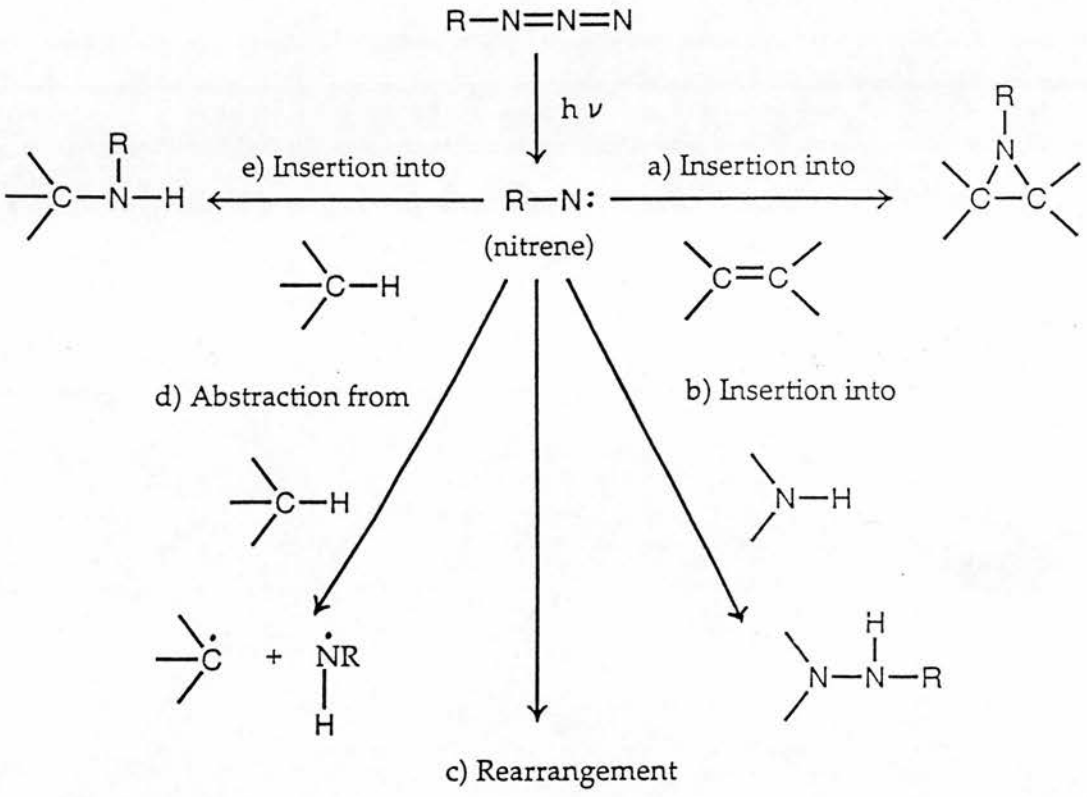


Fig. 1.1 The diversity in reactions of nitrenes. The azido group loses nitrogen by photoactivation and produces nitrene. Because of the extreme instability of nitrene, it shows a great diversity in its reactions (routes a-e). While this may be an advantage if the binding site is barren of a functional group that will react with an alternative labelling agent, it is also a disadvantage : only some of the reactions of nitrene will produce a ligand-binding site covalent bond (routes a, b and e). Rearrangement is one of the reactions that does not result in photolabelling (Cavalla and Neff, 1985).

1984), (±)-7-[^{125}I]iodo-8-hydroxy-3-methyl-1-(4-azidophenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (dopamine D_1 receptor) (Niznik et al., 1988) and [^{3}H]azido-N-methylspiperone (dopamine D_2 receptor) (Niznik et al., 1986) have been used to elucidate the molecular structure and characteristics of receptors.

The major purpose of the work presented in this thesis was to develop suitable radiolabelled ligands in order to study receptors for two CNS neurotransmitters, L-glutamate and 5-hydroxytryptamine.

1.2 GLUTAMATE

Glutamate is a major excitatory neurotransmitter in the mammalian central nervous system (Fonnum, 1984). In 1961, Curtis et al. reported that glutamate was capable of depolarising spinal cord neurones in electrophysiological studies. During the last 25 years, numerous studies demonstrated that glutamate satisfies all criteria for an endogenous transmitter in the mammalian CNS. The uptake of glutamate shows an absolute requirement for sodium (Bennet et al., 1973). L-Glutamate and D- and L-aspartate are taken up by the same uptake mechanism, whereas apart from L-S-sulphocysteine no other endogenous amino acid is taken up to any significant extent by this carrier (Logan and Snyder, 1971; Balcar and Johnson, 1972; Griffiths et al., 1992). Depolarisation methods such as high potassium concentration and electric field stimulation evoke release of glutamate from brain slices or synaptosome preparations in a Ca^{2+}-dependent manner (de Belleroche and Bradford, 1972; Potashner, 1978; Nadler et al., 1977; Nicholls and Sihra, 1986).

Glutamine and 2-oxoglutarate are the important precursors for glutamate synthesis. It has been shown that glutamine is an excellent precursor for the releasable pool of glutamate (Hamberger et al., 1979a,b). Glutamate is synthesised from glutamine by phosphate-activated glutaminase, a mitochondrial enzyme. In addition, glutamate is synthesised from 2-oxoglutarate by aspartate aminotransferase or ornithine ∂-aminotransferase (Fonnum,1984).

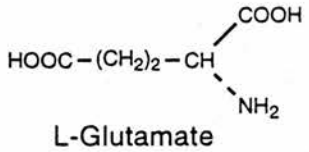
1.2.1 Glutamate Receptors

Glutamate receptors are categorised into two main groups termed ionotropic and metabotropic receptors. Ionotropic glutamate receptors are ligand-gated ion channels named corresponding to selective agonists, N-methyl-D-aspartate (NMDA), DL-α–amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate, and metabotropic receptors are coupled to G-protein (Sladeczek et al., 1988; Monaghan et al., 1989; Watkins et al., 1990) (Table1.1, Fig.1.2,1.3). Activation of NMDA, AMPA and kainate receptors leads directly to opening of ion channels with different permeabilities to Na^+, K^+ and Ca^{2+} ions (Mayer and Westbrook, 1987). The high permeability to Ca^{2+} ions by activation of the NMDA receptor-gated ion channel produces a large increase in intracellular Ca^{2+} concentration which can modulate neuronal excitability (Ozawa et al., 1988). The metabotropic receptors are coupled to multiple second messenger systems that include increases in phosphoinositide hydrolysis, activation of phospholipase D, decreases in cAMP formation, increases in cAMP formation, and changes in ion

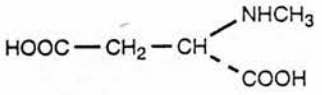
Table 1.1 : Glutamate receptor subtypes

	NMDA				AMPA	Kainate	mGluRs
	competitive site	ion channel	glycine site	polyamine site			
Agonists	L-Glutamate NMDA		Glycine D-Serine D-Alanine	Spermidine Spermine	L-Glutamate Quisqualate AMPA	L-Glutamate Quisqualate Kainate Domoate	L-Glutamate Quisqualate Trans-ACPD AP4 L-CCG-I DCG IV
Antagonists	D-AP5 D-AP7 CPP CGS19755 D-CPPene CGP37849 CGP39551	PCP MK-801 Mg^{2+} Zn^{+}	(+)HA-966 7-Cl-KA L-689,560 L701324 L705022	Ifenprodil SL820715	DNQX CNQX NBQX YM90K		AP3 S-4CPG MCPG
Radioligands	[^{3}H]D-AP5 [^{3}H]CPP [^{3}H]CGS19755 [^{3}H]CGP39653	[^{3}H]PCP [^{3}H]TCP [^{3}H]MK-801	[^{3}H]Glycine [^{3}H]L-689,560		[^{3}H]AMPA [^{3}H]CNQX	[^{3}H]Kainate	[^{3}H]Glutamate
Effector pathways		$Na^{+}/Ca^{2+}/K^{+}$			Na^{+}/K^{+}	Na^{+}/K^{+}	IP_3/DAG cAMP

a)

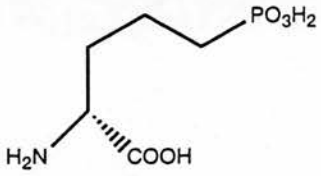


L-Glutamate

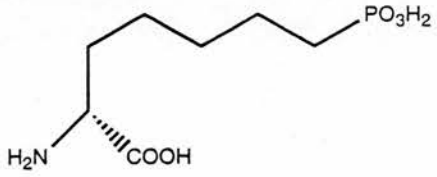


NMDA

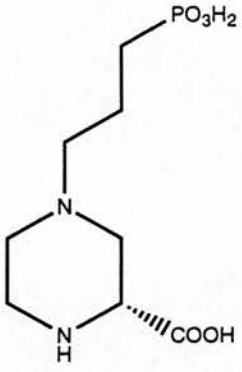
b)



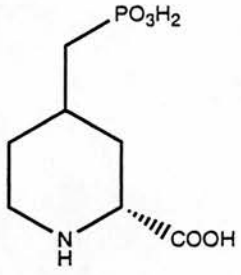
D-AP5



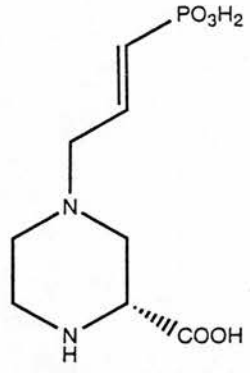
D-AP7



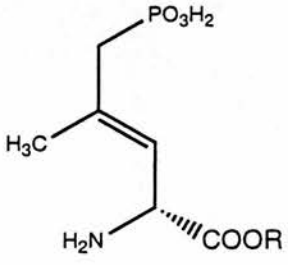
CPP



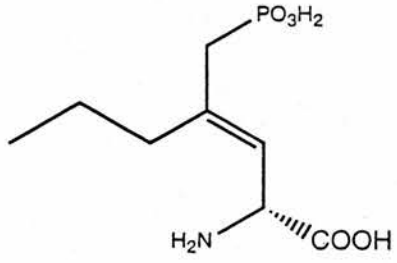
CGS19755



D-CPPene



R=H; CGP37849
R=C2H5; CGP39551



CGP39653

Fig. 1.2 NMDA receptor agonists (a) and competitive antagonists (b).

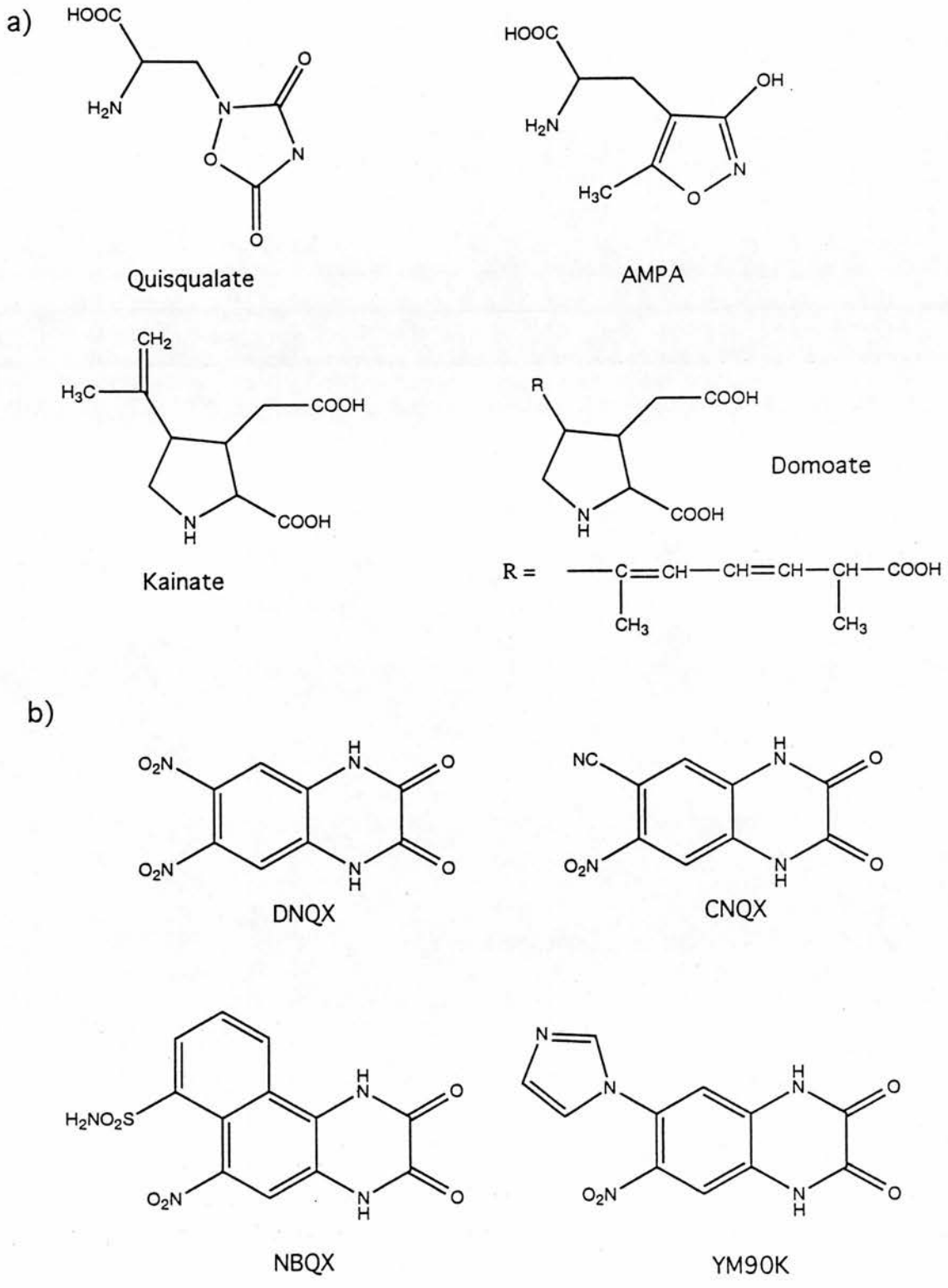


Fig. 1.3 AMPA and kainate receptor agonists (a) and AMPA receptor antagonists (b)

channel function (Sugiyama et al., 1987; Schoepp and Conn, 1993).

1.2.2 NMDA Receptors

The NMDA receptor is the best characterised excitatory amino acid receptor subtype. Agonists such as L-glutamate and NMDA, acting at the NMDA recognition sites, accelerate the influx of Na^+ and Ca^{2+} ions through the ion channel (MacDermott et al., 1986). D-2-Amino-5-phosphonopentanoic acid (D-AP5) (Davies and Watkins, 1982), 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) (Harris et al., 1986) and cis-4-phosphonomethyl-2-piperidine carboxylic acid (CGS19755) (Murphy et al., 1988) act as competitive antagonists at the NMDA receptor recognition sites (Fig.1.2). More recently, the most potent competitive NMDA antagonists, D-(E)-3-(2-carboxypiperazin-4-yl)-1-propenyl-1-phosphonic acid (D-CPPene) (Aebischer et al., 1989) and DL-(E)-2-amino-4-methyl-5-phosphono-3-pentenoic acid (CGP37849) (Fagg et al., 1990) were reported (Fig.1.2). In contrast to D-AP5, CPP and CGS19755, CGP37849 and its carboxyethylester analogue, CGP39551 (Fig.1.2) have significant activity as an anticonvulsant following oral administration (Fagg et al., 1990). Early ligand binding studies with [^{3}H]L-glutamate failed to label the NMDA receptor recognition sites but use of a highly purified membrane preparation or postsynaptic densities gave a successful labelling of the NMDA receptor recognition sites by [^{3}H]L-glutamate (Fagg and Matus, 1984; Monahan and Michel, 1987). On the other hand, the radiolabelled competitive NMDA antagonists [^{3}H]D-AP5

(Olverman et al., 1984; Olverman et al., 1988), [^{3}H]CPP (Olverman et al., 1986) and [^{3}H]CGS-19755 (Murphy et al., 1988) are highly selective for the NMDA-sensitive receptors and useful for labelling the NMDA recognition sites. Sills et al. (1991) reported the radiolabelled 4-propyl analogue of CGP37849, CGP39653 (D,L-(E)-2-amino-4-propyl-5-phosphono-3-pentenoic acid) (Fig.1.2). [^{3}H]CGP39653 is the first competitive antagonist radioligand for the NMDA receptor that exhibits a K_D value in the low nanomolar range (<10 nM). Autoradiographic studies have shown differential distribution profiles in rat brain between [^{3}H]CPP and NMDA-sensitive [^{3}H]L-glutamate binding (Monaghan et al., 1988) and this has been interpreted to suggest the existence of different states of NMDA receptors, agonist-preferring and antagonist-preferring which may be regulated by glycine (Monaghan and Buller, 1994).

Genes encoding two different NMDA receptor subunits have been identified and cloned (Moriyoshi et al., 1991; Monyer et al., 1992; Ishii et al., 1993). The NMDA receptor subunit NMDAR1 (NR1) is a protein of approximately 900 amino acid residues (Moriyoshi et al., 1991). A second subunit NR2 is a large protein of some 1500 amino acids designated NR2A, B, C and D (Monyer et al., 1992; Ishii et al., 1993). By analogy with other ligand-gated ion channel receptors such as GABA and nicotinic acetylcholine, it is likely that the functional NMDA receptors consists of a pentamer containing NR1 and NR2 subunits (Leeson and Iversen, 1994).

1.2.3 AMPA Receptors

AMPA, a structural analogue of L-glutamate, is a potent neuronal excitant when applied electrophoretically near central neurons in the cat spinal cord (Krogsgaard-Larsen et al., 1980). AMPA, quisqualate (Fig.1.3) and glutamate are potent inhibitors of [^{3}H]AMPA binding to rat brain membranes, whereas kainate shows moderate inhibition, whereas NMDA is inactive (Honoré et al., 1982; Honoré, 1989). The affinity of [^{3}H]AMPA for its binding site is stimulated by chaotropic ions (SCN^-) (Honoré and Nielsen, 1985). Honoré and Drejer (1988) have suggested that the [^{3}H]AMPA binding site can exist in two interconvertible forms, and that the effect of SCN^- is to promote formation of the state for which [^{3}H]AMPA has a higher affinity. Selective AMPA receptor antagonists such as 6,7-dinitro-quinoxaline-2,3-dione (DNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and 6-nitro-7-sulphamoyl-benzo(f)quinoxaline-2,3-dione (NBQX) have been reported (Honoré et al., 1988; Sheardown et al., 1990) (Fig.1.3). These compounds inhibit [^{3}H]AMPA binding to rat cortical membranes in submicromolar concentrations. However NBQX is the most selective, 32 times more potent in inhibiting [^{3}H]AMPA binding than [^{3}H]kainate binding (Sheardown et al., 1990). In addition, unlike DNQX and CNQX, NBQX has no or very low affinity for the NMDA receptor and glycine site. Recently it has been demonstrated that 6-(1H-imidazol-1-yl)-7-nitro-2,3(1H,4H)-quinoxalinedione hydrochloride (YM90K) (Fig.1.3) is a selective antagonist for the AMPA receptor, being approximately equipotent with NBQX (Ohmori et al., 1994).

1.2.4 Kainate Receptors

[^{3}H]Kainate has been reported to bind to two populations of sites with high and low affinity (Foster and Fagg, 1984). L-Glutamate, kainate, domoiate (Fig.1.3) and quisqualate are potent inhibitors of [^{3}H]kainate binding (London and Coyle, 1979; Slevin et al., 1983). However domoiate binds preferentially to low affinity kainate sites rather than to high-affinity kainate receptors (Nielsen et al., 1992). In autoradiographic studies, the regional distribution of high affinity [^{3}H]kainate binding sites is unlike those of either AMPA or NMDA receptors, but corresponds well to those brain locations such as hippocampus and cortex that are highly vulnerable to the neurotoxic actions of kainate (Young and Fagg, 1990).

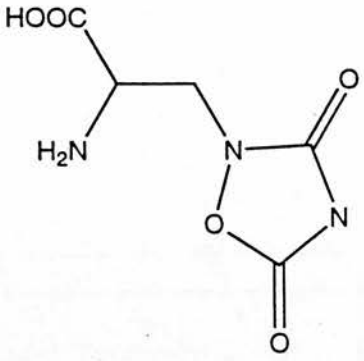
Since a cDNA clone for a functional AMPA-kainate receptor subunit was described (Hollmann et al., 1989), a growing family of related subunits that form functional AMPA-kainate receptor channel complexes has been identified. These subunits can be subclassified into three groups according to their sequence similarity and agonist selectivity. The first consists of four subunits (GluR1, 2, 3, 4) and shows high affinity for AMPA (Boulter et al., 1990; Keinänen et al., 1990), whereas the other two groups represent two different kinds of the kainate selective subunits (GluR5, 6, 7 and KA1, 2) (Bettler et al., 1990; Egebjerg et al., 1991; Herb et al., 1992).

1.2.5 Metabotropic Glutamate Receptors

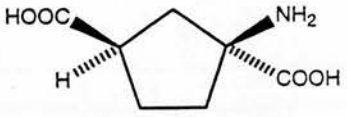
In contrast to ionotropic glutamate receptors (NMDA, AMPA and kainate receptors), metabotropic glutamate receptors (mGluR's) are coupled to cellular effectors via GTP-binding

proteins. Molecular cloning studies have revealed the existence of at least seven subtypes of mGluR's (mGluR1-7) (Abe et al., 1992; Tanabe et al., 1992; Nakanishi, 1992; Okamoto et al., 1994). The seven receptor subtypes can be subdivided into three subgroups according to their sequence similarities : mGluR1 and mGluR5, mGluR2 and mGluR3, and mGluR4, mGluR6 and mGluR7. The mGluR1 and mGluR5 stimulate inositol trisphosphate formation and intracellular Ca^{2+} mobilisation (Abe et al., 1992; Nakanishi, 1992). In contrast, the other mGluR subtypes inhibit the forskolin-stimulated accumulation of intracellular cAMP in an agonist-dependent manner (Nakanishi, 1992; Schoepp and Conn, 1993; Okamoto et al., 1994). Trans-1-amino-1,3-cyclopentanedicarboxylic acid (trans-ACPD) (Fig.1.4), a rigid analog of the extended structure of glutamate, appears to be a selective mGluR agonist. Trans-ACPD stimulates phosphoinositide hydrolysis with high efficacy in rat brain slices (Palmer et al., 1989; Desai and Conn, 1990), cultured striatal neurons and Xenopus oocytes that have been injected with rat brain mRNA (Manzoni et al., 1990). Trans-ACPD comprises a 50 : 50 mixture of 1S,3R-ACPD which is the active constituent and 1R,3S-ACPD (Irving et al., 1990). Other mGluR agonists that are relatively specific include L-2-amino-4-phosphonobutanoate (L-AP4) and 2S,1'S,2'S-2-(2'-carboxycyclopropyl)glycine (L-CCG-I), while quisqualate, ibotenate and (2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl) glycine (DCG-IV) have potent agonist activity at mGluRs but are also highly active at ionotropic glutamate receptors (Ishida et al., 1993; Schoepp and Conn, 1993) (Fig.1.4). However the agonist selectivity is different between receptor subtypes.

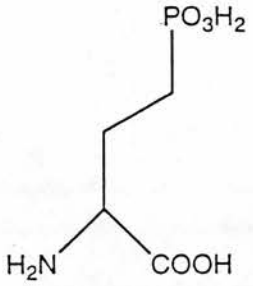
a)



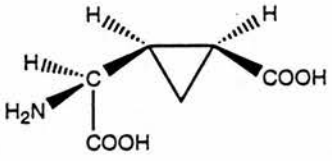
Quisqualate



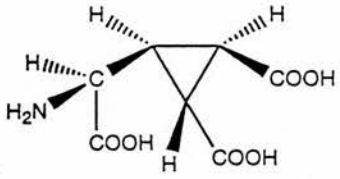
trans-ACPD



AP4

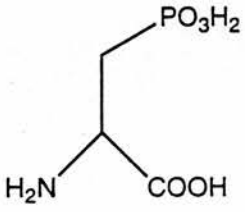


L-CCG-I

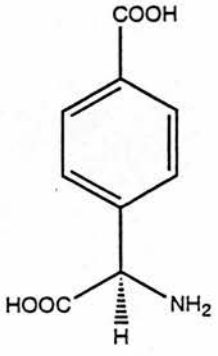


DCG-IV

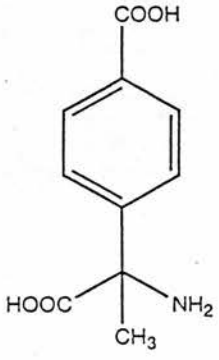
b)



AP3



S-4CPG



MCPG

Fig. 1.4 Metabotropic glutamate receptor agonists (a) and antagonists (b)

L-Glutamate and trans-ACPD are potent agonists for mGluR2/3 whereas quisqualate and L-AP4 are potent agonists for mGluR1/5 and mGluR4 respectively (Nakanishi, 1992; Schoepp and Conn, 1993). L-2-Amino-3-phosphonopropionate (L-AP3) (Fig.1.4) is a potent and selective antagonist which lacks appreciable affinity for ionotropic glutamate receptors (Schoepp et al., 1990). Recently it has been reported that substances structurally related to phenylglycine such as S-4CPG (S-4-carboxyphenylglycine) (Fig.1.4) and MCPG ((RS)-α–methyl-4-carboxyphenylglycine) (Fig.1.4) selectively antagonise the depolarising responses produced by 1S,3R-ACPD in neonatal rat motoneurones, with little or no effect on similar depolarisations produced by the ionotropic glutamate receptor agonists, NMDA and AMPA (Eaton et al., 1993). The mGluR's are known to be implicated in physiological and pathological processes including spatial learning (Richter-Levin et al., 1994) and neuronal damage (Bruno et al., 1994). When injected bilaterally into the lateral ventricles, MCPG disrupts the performance of rats in a spatial learning version of the water maze task in rats, indicating that activation of MCPG-sensitive mGluR's is necessary for spatial learning (Richter-Levin et al., 1994). DCG-IV, a potent agonist of mGluR2/3, protects cultured cortical neurons against excitotoxicity induced either by brief exposure to NMDA or a prolonged exposure to kainate, suggesting a neuroprotective role for mGluR2 or mGluR3 against excitotoxic neuronal death (Bruno et al., 1994).

1.2.6 NMDA Receptor-Ion Channel

The NMDA receptor is a receptor-ion channel complex whose functional activity is modulated by several regulatory sites (Table 1.1, Fig.1.5). The dissociative anesthetics such as phencyclidine (N-(1-phenylcyclo-hexyl)piperidine; PCP) (Fig.1.6) (Honey et al., 1985) and an anticonvulsant dizocilpine ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine; MK-801) (Fig.1.6) (Wong et al., 1986) are non-competitive NMDA antagonists which act by binding to the so-called PCP recognition sites within the ion channel. Both PCP and MK-801 block the stimulation of Ca^{2+} influx by NMDA in cultured neurons (Wroblewski et al., 1987; Yuzaki et al., 1990). L-Glutamate and NMDA potentiate binding of [^{3}H]MK-801 in rat cortical membranes and an excellent correlation was shown between stimulation of [^{3}H]MK-801 binding and inhibition of NMDA-sensitive L-[^{3}H]glutamate binding by excitatory amino acid agonists (Foster and Wong, 1987). An unusual feature of the NMDA receptor is that it is inoperative when target cells are in a resting state, as under such conditions of negative intracellular membrane potential the NMDA receptor ion channel is fully blocked by Mg^{2+} ions in a voltage-dependent manner and this block is removed if the target cell is depolarised by activation of non-NMDA receptors or other excitatory inputs (Mayer et al., 1984; Collingridge and Singer, 1990). Ligand binding studies demonstrate that the interaction of Mg^{2+} with the [^{3}H]MK-801 binding site is non-competitive as Mg^{2+} increases the dissociation rate of [^{3}H]MK-801, indicating that Mg^{2+} sites are distinct from MK-801 binding sites (Reynolds and Miller, 1988). Recently, it has been reported that the

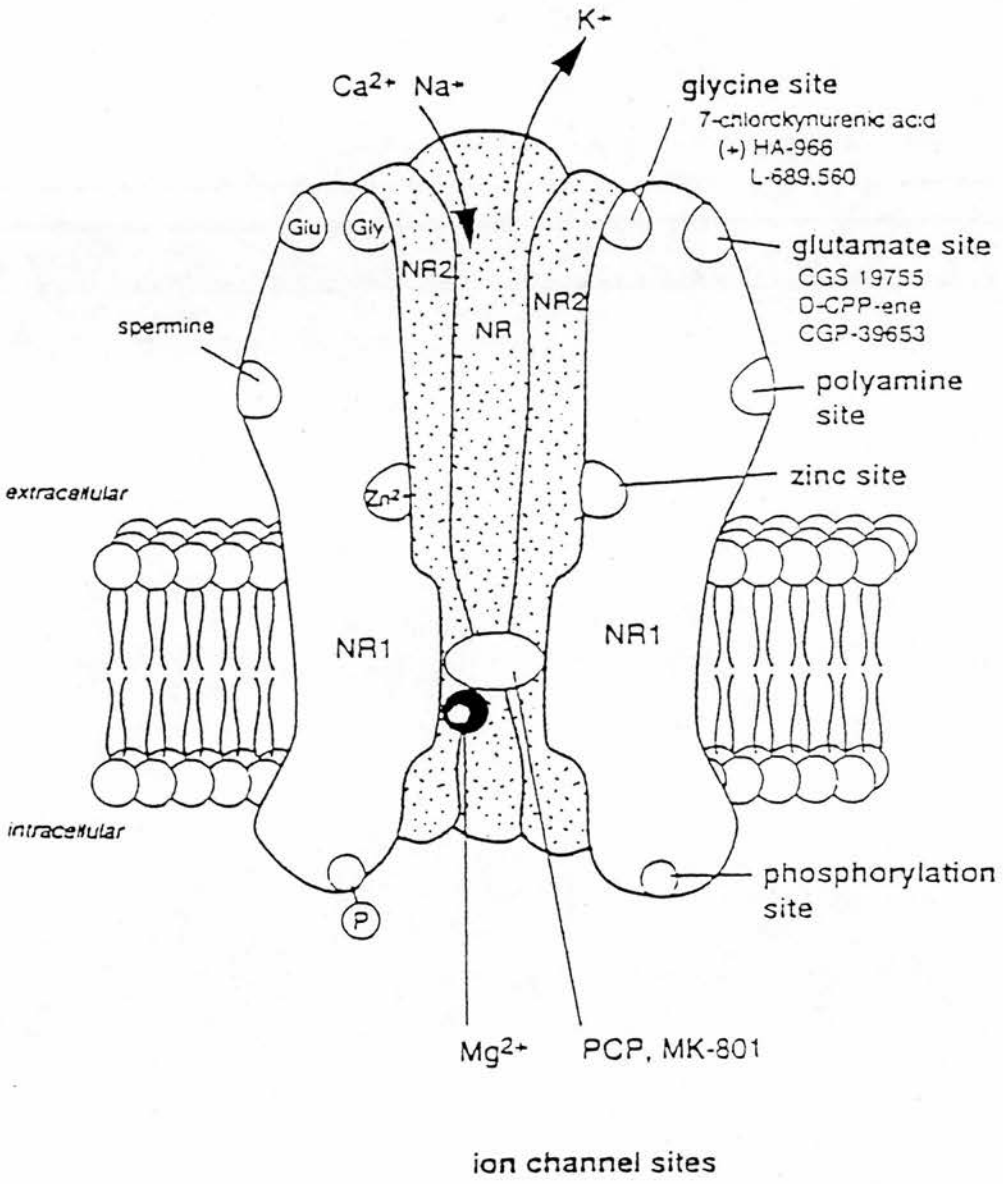


Fig. 1.5 A proposed model for the NMDA receptor-ion channel complex. The NMDA receptor gates a cation channel that is permeable to Ca^{2+}, Na^{+} and K^{+}. The NMDA receptor-ion channel is blocked by PCP, MK-801 and Mg^{2+}, and the complex is regulated at modulatory sites by glycine, Zn^{2+} and polyamines. (adapted from Leeson and Iversen, 1994)

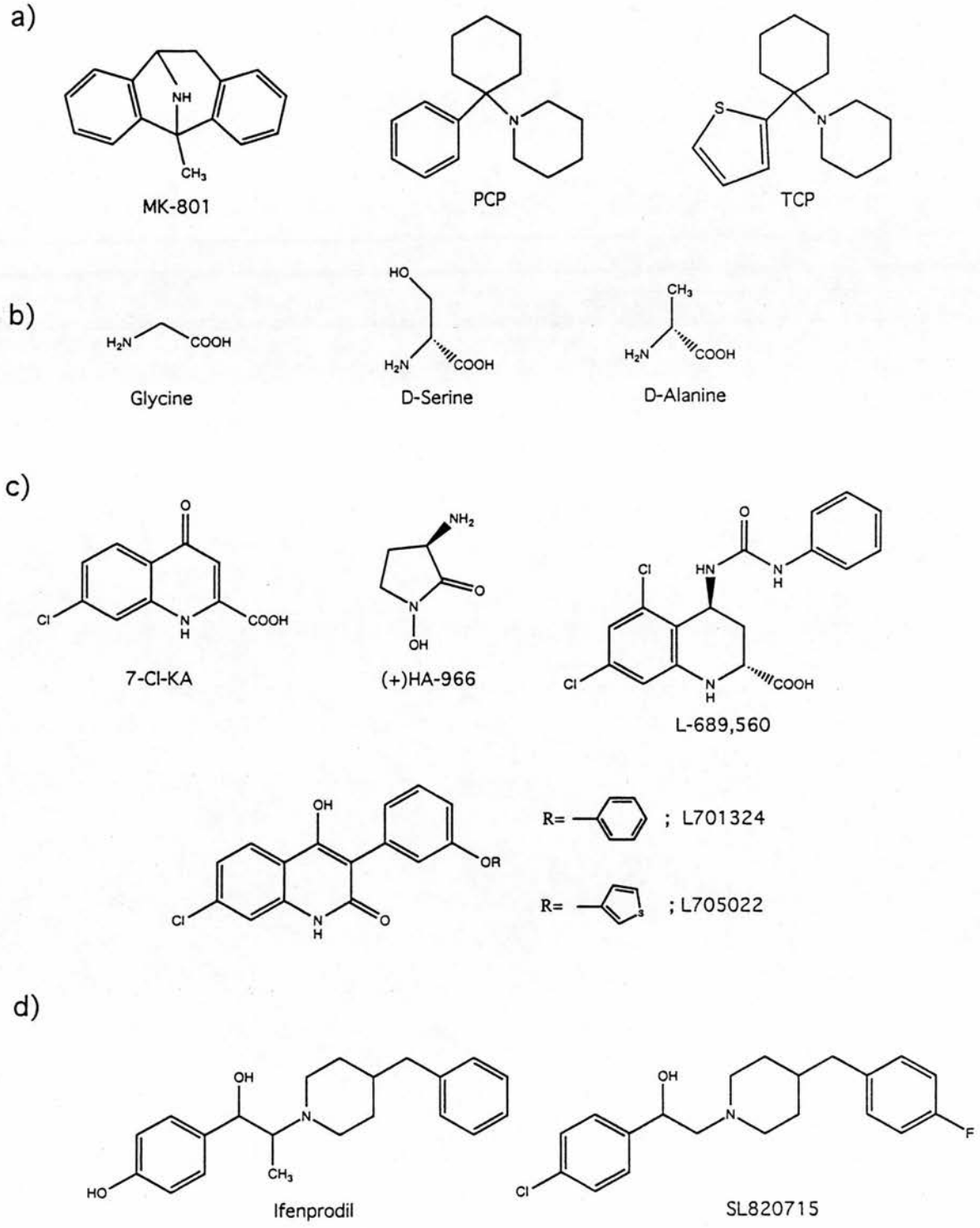


Fig. 1.6 Non-competitive NMDA antagonists (a), glycine agonists (b), glycine antagonists (c) and polyamine antagonists (d).

arylalkylamine spider toxin, argiotoxin 636 may exerts an inhibitory effect on [^{3}H]MK-801 binding to rat brain membranes by binding to the Mg^{2+} sites (Reynolds, 1991).

In addition to these recognition sites, there are at least three other sites which regulate the NMDA receptor-ion channel complex. Firstly, glycine is a positive modulator of the NMDA receptor. Electrophysiological studies have shown that glycine potentiates NMDA-induced responses via a strychnine-insensitive mechanism in cultured cortical neurons (Johnson and Ascher, 1987). Autoradiographic data have demonstrated a similar anatomical distribution for NMDA-sensitive [^{3}H]L-glutamate and strychnine-insensitive [^{3}H]glycine binding (Bristow et al., 1986; Monaghan and Cotman,1985). It has been shown that glycine can enhance binding of [^{3}H]MK-801 and the PCP analogue [^{3}H]TCP; N-(1-thienyl)-cyclohexyl-3,4-piperidine) (Fig.1.6) to rat brain membranes (Wong et al., 1987; Bonhaus et al., 1987). Other monocarboxylic amino acids such as D-serine and D-alanine, are agonists at the glycine site (Fig.1.6) enhancing [^{3}H]TCP binding and inhibiting [^{3}H]glycine binding to rat brain membranes with almost the same potency as glycine (Snell et al., 1988). Kleckner and Dingledine (1988) have shown that agonist occupation of both L-glutamate and glycine recognition sites is required for NMDA receptor activation indicating that glycine functions as a co-agonist. On the other hand, (+)-3-amino-1-hydroxypyrrolid-2-one ((+)HA-966) (Singh et al., 1990), 7-chloro-kynurenic acid (7-Cl-KA) (Kemp et al., 1988), DNQX and CNQX (Kessler et al., 1989) are competitive antagonists for the glycine recognition sites (Fig.1.6). Recently, Foster et al. (1992) showed that 4-trans-2-carboxy-

5,7-dichloro-4-phenylaminocarbonylamino-1,2,3,4-tetrahydroquinoline (L-689,560) (Fig.1.6) inhibits [^{3}H]glycine binding to rat brain membranes with an IC_{50} of 7.8 nM, being substantially more potent than previous compounds such as 7-Cl-KA and DNQX. In addition this compound displays a high degree of selectivity for the glycine site, having IC_{50} values of >10 μM in radioligand binding assays for AMPA and kainate receptors and for the L-glutamate recognition site of the NMDA receptor. The characteristics of [^{3}H]L-689,560 binding to rat brain membranes show a close correlation between the potency of various drugs as inhibitors of [^{3}H]glycine and [^{3}H]L-689,560 binding (Grimwood et al., 1992). Most recently, Kulagowski et al. (1994) reported that two other analogues of L-689,560, 7-chloro-4-hydroxy-3-[3-(phenoxy)phenyl]quinolin-2-(1H)-one (L-701,324) and 7-chloro-4-hydroxy-3-[3-(3-thienyloxy)phenyl] quinolin-2-(1H)-one (L-705,022) are also highly potent and selective glycine antagonists (Fig.1.6). L-701,324 and L-705,022 inhibit [^{3}H]L-689,560 binding to rat brain membranes with an IC_{50} of 2.0 and 1.4 nM respectively, having no activity at amino acid receptors labelled by [^{3}H]AMPA, [^{3}H]kainate and [^{3}H]strychnine at concentrations up to 100 μM. Both compounds have potent anticonvulsant activity in the DBA/2 mouse model with ED_{50} values of below 1 mg/kg following i.p. and p.o. administration, suggesting high oral bioavailability.

Secondly, the divalent cation Zn^{2+} has been shown to prevent NMDA-induced channel activation in neurons in a non-competitive fashion (Westbrook and Mayer,1987). Biochemical studies of ligand binding have found that Zn^{2+} dose-dependently inhibits both the rate of association and

dissociation of [^{3}H]TCP and [^{3}H]MK-801 (Reynolds and Miller, 1988b; Yeh et al., 1990). Recently, it has been demonstrated that a range of tricyclic antidepressants and phenothiazine derivatives inhibit [^{3}H]MK-801 binding and their actions may be mediated by the zinc recognition sites (Reynolds and Miller, 1988a).

Finally, it has been reported that the naturally occurring polyamine compounds such as spermidine and spermine increase the binding of [^{3}H]MK-801 and [^{3}H]TCP to rat brain membranes (Ransom and Stec, 1988; Sacaan and Johnson, 1990). Ransom and Stec (1988) showed an additive effect of spermidine when combined with maximally stimulating concentrations of glutamate and glycine on [^{3}H]MK-801 binding, indicating a distinct site for the polyamines. Ifenprodil and SL820715 ((±)-α-(4-chlorophenyl)-4-[(4-fluorophenyl)methyl]-1-piperidineethanol) (Fig.1.6), developed as potential cerebral antiischaemic agents, have been suggested to exert these effects via an interaction with the polyamine sites (Carter et al., 1989; Carter et al., 1990). Although the polyamine site is shown to be extracellular in fig.1.5, its location on the NMDA receptor is still not clear. It has been shown that spermidine and spermine enhance NMDA-induced currents in striatal and hippocampal cultured neurones in electrophysiological studies using whole cell patch-clamp recordings (Sprosen and Woodruff, 1990; Williams et al., 1990). Thus the NMDA receptor complex has been shown to have at least six distinct modulatory sites.

Some investigators have demonstrated the labelling of the NMDA receptor protein by photoaffinity ligands, azido analogues of PCP and MK-801 (Haring et al., 1986; Sonders et al., 1990).

The labelling of several polypeptides (Mr 90,000-95,000) has been shown using [^{3}H]meta-azido-PCP in rat brain membranes (Haring et al., 1986). Sonders et al. (1990) reported that [^{3}H]3-azido-MK-801 labels a single protein band using guinea pig brain membranes (Mr 120,000) and rat brain membranes (Mr 115,000). The molecular size of the cloned NMDA receptor subunit (NR1) (Mr 105,500) is consistent with that for the polypeptide identified by [^{3}H]3-azido-MK-801.

1.2.7 NMDA Receptor-mediated Physiological and Pathological Processes

The NMDA receptor is known to be implicated in a number of physiological and pathological processes such as long-term potentiation (Artola and Singer, 1987), learning and memory (Collingridge, 1987), ischaemic neuronal damage (Gill et al., 1987; Boast et al., 1988) and in several CNS disorders such as epilepsy (Meldrum, 1985), Huntington's disease (Foster et al., 1985) and Alzheimer's disease (Greenamyre et al., 1987). Low frequency transmission is mediated by glutamate acting principally via AMPA receptors, since NMDA receptor operated channels are blocked by Mg^{2+}. During high frequency transmission the Mg^{2+} block is removed and Ca^{2+} enters neurones via activated NMDA receptor channels (Collingridge and Singer, 1990). Long-term potentiation (LTP) in the CA1 region of the hippocampus is a widely studied model of activity-dependent change in synaptic efficacy. It is usually induced by a period of high frequency stimulation and is manifest as an increase in the size of the synaptic response evoked by low frequency stimulation of the Schaffer collateral-commissural pathway

(Collingridge et al., 1983). A brief high frequency stimulation of presynaptic neurons facilitates synaptic efficacy at postsynaptic neurons and this phenomenon can be observed to last for several days (Bliss and Lomo, 1973). AP5 and PCP block induction of LTP in the hippocampus, indicating that the NMDA receptors are responsible for mediating LTP (Collingridge et al., 1983; Stringer et al.,1983). Morris et al. (1986) demonstrated that AP5 impairs spatial learning in rats in the water maze test in addition to inhibiting LTP indicating that LTP may be closely associated with certain forms of learning and memory.

PCP is a potent psychotomimetic agent that produces a psychosis resembling schizophrenia. The similarity in symptoms between the PCP-induced psychosis and schizophrenia has led to the hypothesis that PCP is a better drug than amphetamine for the study of schizophrenia (Allen and Young, 1978). PCP and MK-801 produce symptoms resembling the action of high doses of amphetamine in experimental animals (Freed et al., 1980; Clineschmidt et al., 1982). Tanii et al. (1991) showed that intracerebro-ventricular injections of agonists for the NMDA receptor glycine site, such as D-serine and D-alanine, inhibit PCP-induced hyperactivity in rats. Moreover it has been demonstrated that D-serine antagonises PCP- and MK-801-induced stereotyped behaviour and ataxia in rats (Contreras, 1990). These studies may indicate that positive modulators of NMDA receptor function are a useful supplement in the therapy of psychotic disorders.

Brain neuronal degeneration may be a result of excessive release of excitatory amino acids following ischaemia (Hagberg et al., 1985). Short periods of cerebral ischaemia produce

delayed neuronal death in the hippocampus of gerbils (Kirino, 1982). The hippocampal CA_1 and CA_2 pyramidal neurons are most vulnerable to cerebral ischaemia and have a large number of NMDA receptors (Monaghan and Cotman, 1985). It has been shown that competitive NMDA receptor antagonists such as CPP and CGS19755 protect against hippocampal damage following cerebral ischaemia in gerbils (Boast et al., 1988). The potent non-competitive NMDA antagonist MK-801 is also effective in the global ischaemia model in gerbils (Gill et al., 1987). In addition, these NMDA receptor antagonists can protect against selective neuronal loss in the CA_1 region of hippocampus following transient ischaemia in rats (Swan and Meldrum, 1990). Recently it was suggested that the neuroprotective effect of MK-801 in gerbils is due to drug-induced hypothermia rather than NMDA receptor antagonism (Buchan and Pulsinelli, 1990). However, Gill and Woodruff (1990) showed that MK-801 is still efficacious in the gerbil global ischaemia model where core body temperature is maintained at 37°C for 24 hr following ischaemia. These results suggest a substantial involvement of NMDA receptors in delayed neuronal death in animal models. The most widely used model of stroke is middle cerebral artery (MCA) occlusion in rodents. MK-801 has been reported to reduce ischaemic cortical damage in a rat MCA occlusion model (Park et al., 1988). In the rat MCA occlusion model, a marked increase in the extracellular concentrations of excitatory amino acids such as L-glutamate and L-aspartate are observed in the brain during and shortly after a period of transient ischaemia (Butcher et al., 1990). These results indicate that the cerebroprotective effect of MK-801 in the rat focal ischaemia model results from NMDA

receptor antagonism. Electrophysiological and ligand binding studies have demonstrated that MK-801 acts in a use-dependent mode, i.e. the degree of NMDA receptor blockade is increased in the presence of NMDA receptor agonists (Huettner and Bean, 1988; Foster and Wong, 1987). This property is expected to give an advantage over competitive antagonists in that the drug may show little blocking action on the physiological functions of NMDA receptors but it may selectively block the NMDA receptor ion channels under ischaemia conditions. Thus non-competitive NMDA antagonists might be useful in the treatment of human ischaemic neuropathologies.

Chapman et al. (1983) reported that [^{3}H]2-amino-7-phosphonoheptanoic acid (AP7), a potent competitive NMDA antagonist, is taken up into mouse brain following intraperitoneal injection and that the time course for the uptake of AP7 parallels the period of anti-convulsant protection. The characterisation of in vivo receptor binding with [^{3}H]MK-801 in the mouse brain was reported by Price et al. (1988a,b). Furthermore it has been demonstrated that radioiodinated [^{125}I](+)-3-iodo-MK-801 may be useful as a radioligand for in vivo imaging of the NMDA receptor complex (Gibson et al., 1992).

Like MK-801, an isoquinoline derivative FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride) (Fig.1.7) is a novel non-competitive NMDA antagonist and has antiischaemic and anticonvulsive effects in animals (Nakanishi et al., 1994; Katsuta et al., 1995).

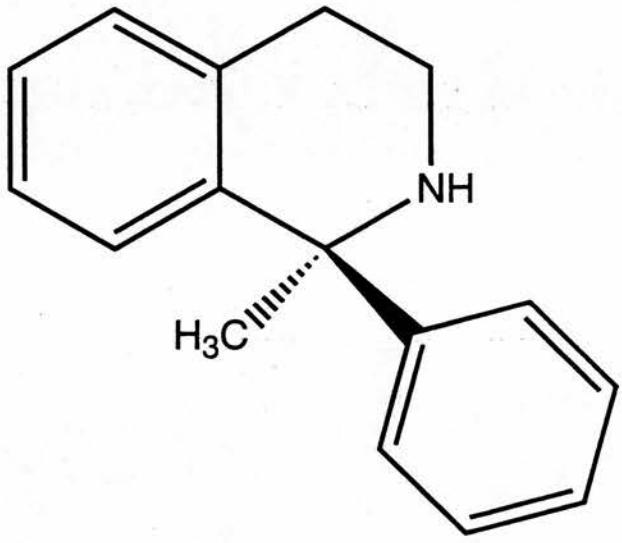


Fig. 1.7 Chemical structure of FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride)

The aim of the work presented in chapter 2 was therefore firstly to characterise FR115427 using in vitro [^{3}H]MK-801 binding to rat brain membranes, and secondly to synthesise and purify [^{3}H]FR115427 and to examine its in vivo distribution in rats.

1.3 5-HYDROXYTRYPTAMINE (5-HT)

Rapport et al. (1947) reported the existence of a vasoactive substance in serum. Shortly after, this substance was identified as 5-HT (Rapport, 1949). Twarog and Page (1953) demonstrated that 5-HT exists in mammalian brain. The biosynthesis and metabolism of 5-HT follow the pathway shown in fig.1.8. Tryptophan is converted into 5-hydroxytryptophan by the action of tryptophan hydroxylase and then into 5-HT by L-aromatic acid decarboxylase (Udenfriend et al., 1953; Freedland et al., 1961). Degradation of 5-HT occurs mainly by metabolism by monoamine oxidase and subsequent transformation of the aldehyde by aldehyde dehydrogenase into 5-hydroxyindoleacetic acid (5-HIAA) (Leeper et al., 1958; Smith and Wortis, 1960). Under normal physiological conditions, tryptophan hydroxylase may only be half saturated (Carlsson and Lindqvist, 1972). Consequently, changes in the levels of its substrate, tryptophan, will trigger alterations in 5-HT synthesis and metabolism. Tryptophan circulates in the blood in free form (normally 10-20%) and albumin-bound form (McMenamy et al., 1957) and is taken up into the brain by a saturable and stereospecific carrier which also transports other large neutral amino acids such as phenylalanine, tyrosine and leucine into the

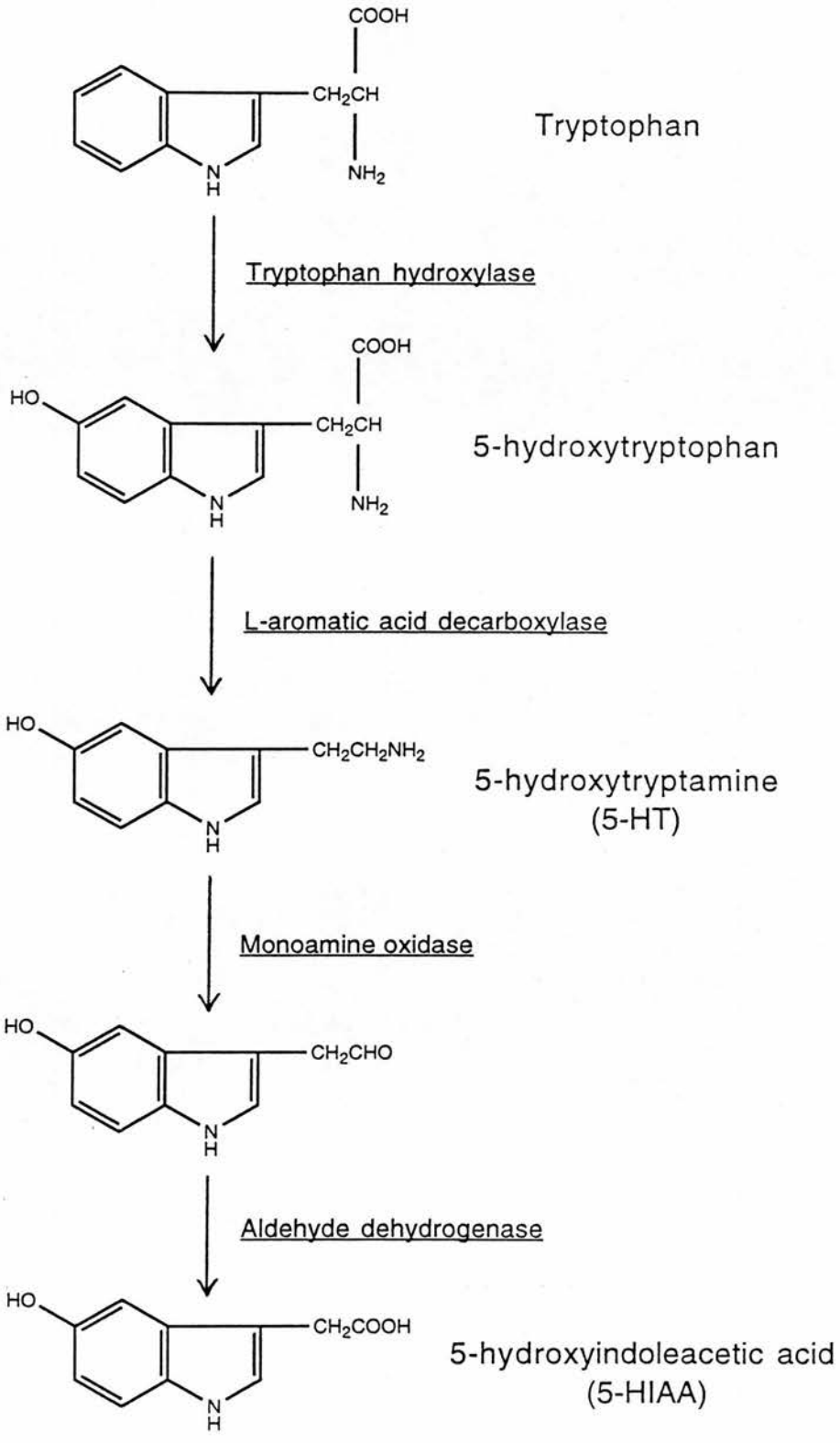


Fig. 1.8 Biosynthesis and metabolism of 5-HT

cerebrum (Fernstrom and Wurtman, 1972; Pardridge, 1977). Exocytotic release of 5-HT is regulated by processes such as synthesis of 5-HT and neuronal firing rate. For example, pretreatment with the tryptophan hydroxylase inhibitor p-chlorophenylalanine (PCPA) decreases p-chloroamphetamine (PCA)-induced 5-HT release in striatum (Marsden et al., 1979). Electrical stimulation of raphe nuclei, where serotonergic cell bodies are located, accelerates the release of 5-HT from nerve terminals (Marsden et al., 1979; Hery and Ternaux, 1981).

1.3.1 5-HT_1 Receptors

Evidence for the existence of serotonergic receptors was first presented by Gaddum and Picarelli (1957). They described two types of receptors (D and M receptors) controlling muscle contraction in order to account for two distinct effects of 5-HT in isolated guinea pig ileum. The classification and identification of multiple 5-HT receptors has been largely based on radioligand binding studies (Table 1.2) but more recently, really, superceded by molecular biological data (Humphrey et al., 1993). 5-HT receptors were first classified as 5-HT_1 and 5-HT_2 by Peroutka and Snyder (1979). 5-HT_1 sites are labelled by [^{3}H]5-HT with nanomolar affinity, whereas 5-HT_2 sites are labelled by [^{3}H]spiroperidol in rat cortical membranes. The 5-HT_1 receptor was subdivided further into 5-HT_{1A} (high affinity for spiroperidol) and 5-HT_{1B} (low affinity for spiroperidol) (Pedigo et al., 1981).

5-HT_{1A} receptors have been more selectively labelled with [^{3}H]8-hydroxy-2-(di-n-propylamino)-tetralin(8-OH-DPAT) (Fig.1.9) (Hall et al., 1985). The receptor binding profile shows

Table 1.2 : 5-HT receptor subtypes

	5-HT$_{1A}$	5-HT$_{1B}$	5-HT$_{1D}$	5-HT$_{1E}$	5-HT$_{1F}$	5-HT$_{2A}$	5-HT$_{2B}$	5-HT$_{2C}$	5-HT$_3$	5-HT$_4$	5-HT$_{5A,B}$	5-HT$_6$	5-HT$_7$
Previous names						D, 5-HT$_2$		5-HT$_{1C}$	M				
Agonists	8-OH-DPAT Buspirone Ipsapirone		Sumatriptan			α-Methyl -5HT	α-Methyl -5HT	α-Methyl -5HT mCPP	2-Methyl -5HT	5-Methoxy -tryptamine Cisapride BIMU8			
Antagonists	WAY-100135 WAY-100635		GR127935			Ketanserin Ritanserin Cyproheptadine	SB200646A	SB200646A Mesulergine	GR65630 MDL72222 CS205-930 Granisetron Zacopride Ondansetron	SDZ205557 DAU6285 GR113808			
Radioligands	[^{3}H]8-OH-DPAT	[^{125}I]Iodo-cyanopindolol	[^{3}H]5-HT	[^{3}H]5-HT	[^{3}H]LSD	[^{3}H]Ketanserin		[^{3}H]Mesulergine [^{3}H]LSD	[^{3}H]GR65630 [^{3}H]Granisetron [^{3}H]Zacopride	[^{3}H]GR113808	[^{3}H]LSD	[^{3}H]LSD	[^{3}H]LSD
Effector pathways	cAMP ↓ K$^+$ channel	cAMP ↓	cAMP ↓	cAMP ↓	cAMP ↓	IP$_3$/DAG	IP$_3$/DAG	IP$_3$/DAG	Internal cation channel	cAMP ↑	Unkown	cAMP ↑	cAMP ↑

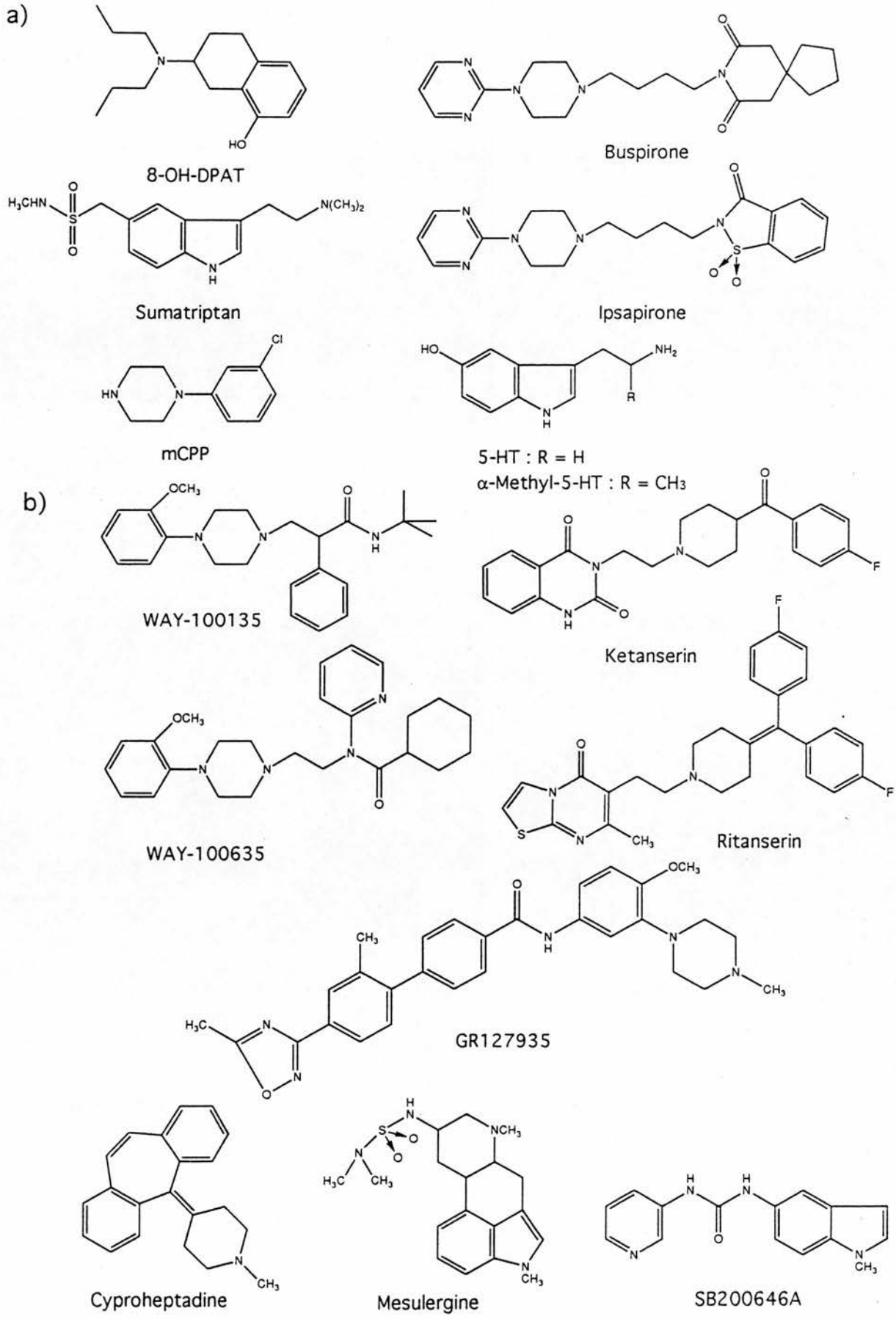


Fig. 1.9 5-HT1 and 5-HT2 agonists (a) and antagonists (b)

that 8-OH-DPAT displays high affinity for central 5-HT$_{1A}$ receptors and weak or negligible affinity for other 5-HT receptor subtypes. Another class of 5-HT$_{1A}$-active compounds are the pyrimidinyl-piperazines such as buspirone and ipsapirone (Fig.1.9) (Traber and Glaser, 1987). In contrast to buspirone, ipsapirone displays weak affinity for dopamine D$_2$ receptors. 5-HT$_{1A}$ receptors are highly localised in hippocampus and cerebral cortex and also in the raphe nuclei which contain serotonergic cell bodies (Pazos and Palacios, 1985; Waeber et al., 1989). In raphe nuclei, the 5-HT$_{1A}$ receptors act as somatodendritic autoreceptors regulating the firing of serotonergic neurons. 5-HT$_{1A}$ agonists such as buspirone and ipsapirone have anxiolytic properties in man (Goldberg and Finnerty, 1979; Glaser, 1988). The effect of buspirone is probably to decrease 5-HT function because administration of buspirone decreases raphe cell activity (Van der Maelen and Wilderman, 1984). The selective 5-HT$_{1A}$ receptor ligand shown to display unequivocal 5-HT$_{1A}$ receptor antagonist properties at both presynaptic and postsynaptic 5-HT$_{1A}$ receptors is N-tert-butyl-3-4(2-methoxy-phenyl-piperazin-1-yl-2-phenylpropanamide dihydrochloride (WAY-100135) (Fig.1.9) (Fletcher et al., 1991; Jones and Haskins, 1991). WAY-100135 has high affinity for the 5-HT$_{1A}$ binding site (IC_{50} = 34 nM) and shows at least 70-fold selectivity against other 5-HT, noradrenergic and dopaminergic sites (Fletcher et al., 1991). Anxiolytic properties of WAY-100135 have been demonstrated in the mouse light/dark box model of anxiety and in the rat potentiated acoustic startle model (Fletcher et al., 1992a, 1992b). Recently, Fletcher et al. (1994) reported that N-(2-

(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridyl) cyclohexane-carboxamide trihydrochloride (WAY-100635) (Fig.1.9) is a new selective 5-HT$_{1A}$ receptor antagonist with higher affinity than WAY-100135.

5-HT$_{1B}$ sites are labelled with [^{125}I]iodo-cyanopindolol in the presence of isoprenaline to block β-adrenergic receptor sites (Hoyer et al., 1985). Selective pharmacological tools to characterise 5-HT$_{1B}$ receptors are not yet available. The 5-HT$_{1B}$ receptor subtypes are observed in rat and mouse brain but not in human brain (Pazos and Palacios, 1985; Hoyer et al., 1985; Martial et al., 1989). The highest density of 5-HT$_{1B}$ receptors has been found in substantia nigra, globus pallidus, dorsal subiculum and superior colliculi. A major function of the 5-HT$_{1B}$ receptors is the control of 5-HT release from the serotonergic neuron terminals (Engel et al., 1986). 5-HT$_{1B}$ receptors are also located on nonserotonergic terminals where they inhibit the release of other neurotransmitter such as acetylcholine and dopamine (Maura and Raiteri, 1986; Benloucif and Galloway, 1991). Thus 5-HT$_{1B}$ receptors may play an important role in the control of CNS function by modulating the release of neurotransmitters.

On the basis of [^{3}H]5-HT binding studies with bovine brain in the presence of drugs masking 5-HT$_{1A, 1B}$ and $_{1C}$ subtypes, the existence of the 5-HT$_{1D}$ receptor subtype was proposed by Heuring and Peroutka (1987). Human genes encoding two 5-HT$_{1D}$ receptor subtypes, 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$, have been described and the 5-HT$_{1D\beta}$ receptor has been shown to exhibit very high sequence homology (93% overall; 96% in the transmembrane regions) with the rodent 5-HT$_{1B}$ receptor but

5-HT$_{1D\beta}$ and 5-HT$_{1B}$ receptors exhibit completely different pharmacological profiles (Adham et al., 1992). A single amino acid substitution of Thr to Asn at position 355 in the seventh transmembrane region accounts for the profound differences in pharmacology displayed by these two receptors (Parker et al., 1993). GR43175 (3-(2-dimethylamino)ethyl-N-methyl-1H-indole-5-methane- sulfonamide, sumatriptan) (Fig.1.9) has been reported as one of the most selective agonists for 5-HT$_{1D}$ sites although this drug recognises 5-HT$_{1B}$ sites with a similar affinity and 5-HT$_{1A}$ sites with a slightly lower affinity (Peroutka and McCarthy, 1989). Since sumatriptan has an antimigraine effect in human, the 5-HT$_{1D}$ receptor may prove to be an important target in the acute treatment of migraine (Doenicke et al., 1988). Recently, N-(4-methoxy-3-(4-methyl-1-piperazinyl)phenyl)-2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)(1,1-biphenyl)-4-carboxamide (GR127935) (Fig.1.9) has been identified as a very potent and selective 5-HT$_{1D}$ receptor antagonist (Skingle et al., 1993). GR127935 inhibits binding to 5-HT$_{1D}$ receptor (pKi 8.5) and shows low or moderate affinity at 5-HT$_{1A}$ (6.9), 5-HT$_{1C}$ (6.4) and 5-HT$_2$ receptors (6.6), and antagonises sumatriptan-induced contractions in dog basilar arterial rings (Skingle et al., 1993).

5-HT$_{1C}$ sites were initially identified using [^{3}H]5-HT, [^{3}H]mesulergine and [^{3}H]lysergic acid diethylamide (LSD) (Pazos et al., 1985). As in the case of 5-HT$_{1B}$ receptors, truly selective 5-HT$_{1C}$ ligands have not yet been reported. The serotonergic drugs that are antagonistic at 5-HT$_2$ receptors generally are also recognised by 5-HT$_{1C}$ sites with high affinity. The most frequently used 5-HT$_{1C}$ ligand is mesulergine (Fig.1.9)

which has a high affinity for 5-HT$_{1C}$, 5-HT$_2$, and D$_2$ receptors. It has been demonstrated that there is a high concentration of 5-HT$_{1C}$ receptors in the choroid plexus of pig and humans (Pazos et al., 1985; Hoyer et al., 1986b). Pazos et al. (1985) suggested that 5-HT$_{1C}$ sites may play a role in the regulation of cerebrospinal fluid (CSF) production and in cerebral circulation because the main physiological role of the choroid plexus is the control of the volume and composition of CSF. In rats, several in vivo responses to the putative 5-HT$_{1C}$ receptor agonist, 1-(3-chlorophenyl)piperazine (mCPP) (Fig.1.9) have been suggested to be mediated by 5-HT$_{1C}$ receptor activation. These include mCPP-induced hypolocomotion (Kennett and Curzon, 1988a), hypophagia (Kennett and Curzon, 1988b) and anxiety (Kennett et al., 1989). In addition it has been reported that mCPP produces anxiety in healthy volunteers and to a greater extent in patients with either panic disorder or obsessive compulsive disorder (Kennett, 1993). Therefore specific 5-HT$_{1C}$ receptor antagonists may be anxiolytics.

5-HT$_{1E}$ and 5-HT$_{1F}$ receptor cDNAs have been cloned and the recombinant proteins classified as 5-HT$_1$ receptor subtypes on the basis of their amino acid homology and their negative coupling to adenylyl cyclase in cell lines (Amlaiky et al., 1992; Adham et al., 1993).

1.3.2 5-HT$_2$ Receptors

5-HT$_2$ binding sites have been characterised by the first potent and still most frequently used agent, [^{3}H]ketanserin (Fig.1.9) (Leysen et al., 1982). There is growing evidence that blockade of 5-HT$_2$ receptors improves schizophrenic symptoms

and that 5-HT systems are involved in its pathogenesis. A selective 5-HT_2/5-HT_{1C} receptor antagonist, ritanserin (Fig.1.9), either alone or in combination with a neuroleptic, reduces negative symptoms in patients with schizophrenia (Gelders et al., 1986). Silver et al. (1989) reported that the mixed 5-HT_1/5-HT_2 antagonist, cyproheptadine (Fig.1.9), is effective in alleviating both positive and negative symptoms in an open pilot study. These beneficial effects of the 5-HT receptor antagonist in schizophrenia may suggest that 5-HT receptor function is altered in some schizophrenic patients.

Recently 5-HT_2 receptors have been sub-classified into 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptor subtypes (Humphrey et al., 1993). The 5-HT_{2A} receptor subtype refers to the classical 5-HT_2 receptor. The 5-HT_{2B} mRNA transcript has been identified in rat stomach fundus (Foguet et al., 1992) and in the small intestine, kidney, heart and cerebellum of the mouse (Loric et al., 1992), but not in rat brain (Foguet et al., 1992). The 5-HT_{1C} receptor is now recognised as belonging to the 5-HT_2 receptor family on the basis of marked amino acid sequence homology (Saltzman et al., 1991) and also because it is coupled to phospholipase C rather than to adenylate cyclase. Therefore the 5-HT_{1C} receptor has been renamed as the 5-HT_{2C} receptor. (N-(1-methyl-5-indolyl)-N'-(3-pyridyl)urea hydrochloride (SB200646A) (Fig.1.9) is the first reported antagonist with selectivity for the 5-HT_{2C} over the 5-HT_{2A} receptor (Forbes et al., 1993). It shows 50-fold selectivity for the 5-HT_{2C} over the 5-HT_{2A} receptor, and 80-fold or greater selectivity over other neurotransmitter receptors tested (Forbes et al., 1993; Kennett et al., 1994). However SB200646A is also equipotent at the

5-HT2B receptor in rat stomach fundus. This compound can block mCPP-induced hypolocomotion, hypophagia and anxiogenesis, indicating that 5-HT2C/2B receptor blockade induces anxiolysis (Kennett et al., 1994).

1.3.3 5-HT3 Receptors

5-HT1-like receptors have been found to modulate adenylate cyclase activity (Bouhelal et al., 1988; De Vivo and Maayani, 1986; Schoeffter et al., 1988), whereas 5-HT2 receptors stimulate phosphatidyl inositide hydrolysis (Conn and Sanders-Bush,1984; Hoyer et al., 1988). In contrast, the 5-HT3 receptor, formerly the classical M-type receptor of Gaddum and Picarelli (1957), appears to be directly linked to a cation channel selectively permeable K^+ and Na^+ (Derkach et al., 1989). Specific 5-HT3 binding sites in the CNS were initially demonstrated by Kilpatrick et al. (1987) using [3H]GR65630 (3-(5-methyl-1H-imidazol-4-yl)-1-(1-methyl-1H-indol-3-yl)-1-propanone) (Fig.1.10). Since the first published 5-HT3 antagonist, 1-αH-3α-5αH-tropan-3-yl-3,5-dichlorobenzoate (MDL72222), many potent 5-HT3 antagonists such as (3-α-tropanyl)1H-indole-3-carboxylic acid ester (ICS205-930, tropisetron), (endo-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-1-methyl-imidazole-3-carboxamide (BRL43694, granisetron), 4-amino-N-(1-aza-bicyclo[2.2.2]oct-3-yl)-5-chloro-2-methoxy-benzamide (zacopride) and (±)-1,2,3,9-tetrahydro-9-methyl-3[(2-methyl-1H-imidazol-1-yl)methyl]-4H-carbazol-4-one (GR38032, ondansetron) have been reported (Watling, 1988) (Fig.1.10). These compounds display subnanomolar affinity for 5-HT3 receptors, whereas affinity for other 5-HT receptor

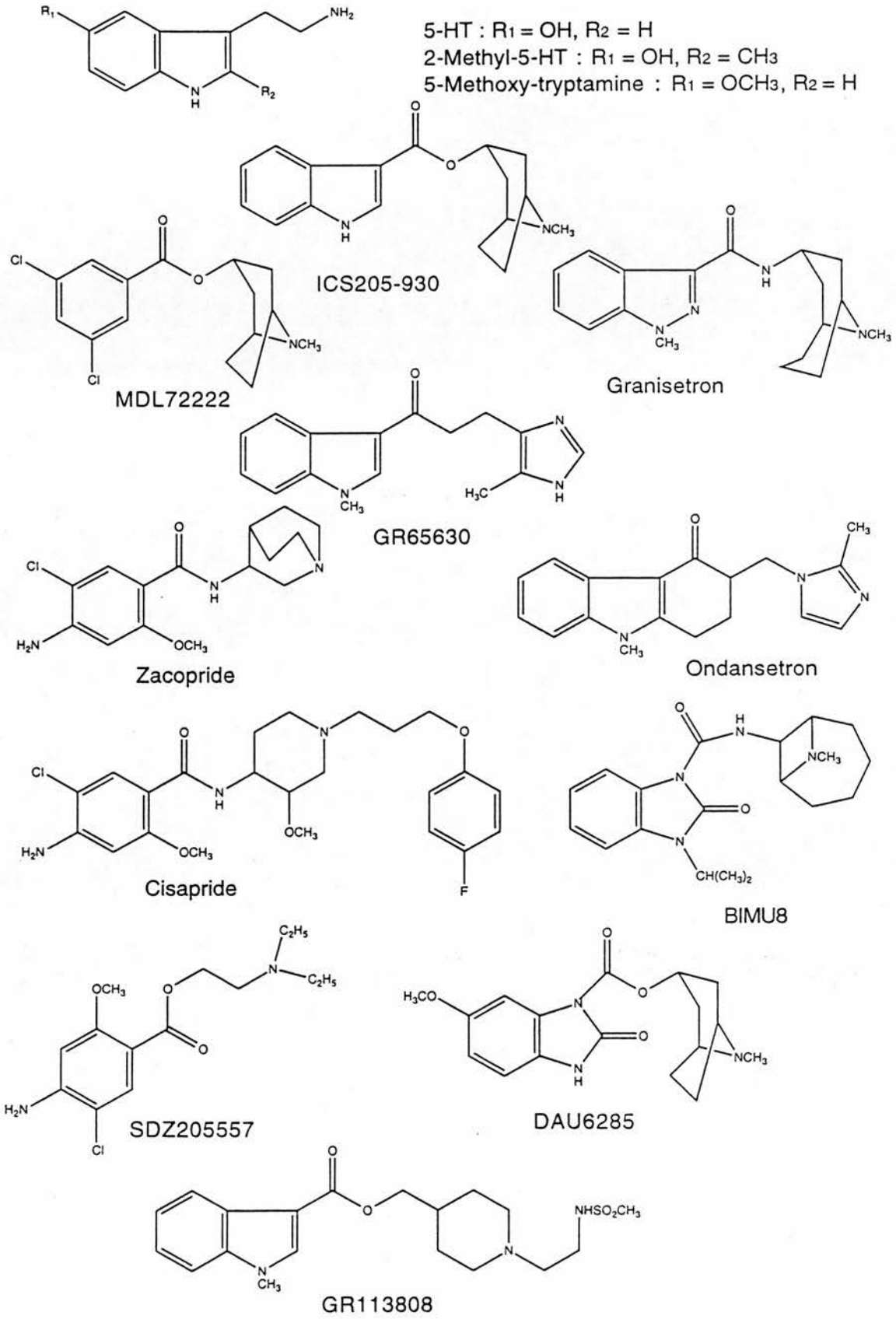


Fig. 1.10 5-HT3 and 5-HT4 agonists and antagonists

subtypes is negligible. The regional distribution of 5-HT3 receptors in the CNS has been studied in various species such as mouse, rat, ferret, rabbit and human brain (Kilpatrick et al., 1987, 1989; Barnes et al., 1989). These studies have demonstrated that 5-HT3 receptors occur in various brain regions such as entorhinal cortex, frontal cortex, hippocampus and thalamus. The highest density of 5-HT3 receptors is observed in the area postrema which is involved in the emesis mechanism. Clinical studies have established that 5-HT3 antagonists are potent inhibitors of cytotoxic drug-induced vomiting in humans (Leibundgut and Lancranjan, 1987; Carmichael et al., 1988; Miln and Heel, 1991). Costall et al. (1990) proposed that 5-HT3 receptors in the brain contribute to the control of behaviour. For example 5-HT3 antagonists can inhibit the hyperactivity caused by the infusion of dopamine into the limbic areas of rat and marmoset brain and have anxiolytic properties in experimental anxiety models such as the social interaction test and the light/dark exploration test (Costall et al., 1987; Costall et al, 1989; Tyers et al., 1987).

1.3.4 5-HT4 Receptors

The existence of an additional 5-HT receptor, the 5-HT4 receptor, which is positively coupled to adenylyl cyclase activity was demonstrated in primary neuronal cell cultures of mouse embryo colliculi (Dumuis et al., 1988). Dumuis et al. (1988, 1989, 1991) showed that 5-methoxy-tryptamine, cis-4-amino-5-chloro-N-{1-[3-(4-fluoro-phenoxy)propyl]-3-methoxy-4-piperidinyl}-2-methoxybenzamide (cisapride), zacopride and (endo-N-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-3-

isopropyl-2-oxo-1H-benzimidazol-1-carboxamide hydrochloride (BIMU8) (Fig.1.10) have 5-HT$_4$ receptor agonist activity. Until recently, the only 5-HT$_4$ receptor antagonist was ICS205-930, a 5-HT$_3$ antagonist with a relatively low affinity and poor selectivity for the 5-HT$_4$ receptor (Craig and Clarke, 1990). It has been shown that the benzamide derivative, 2-methoxy-4-amino-5-chloro-benzoic acid 2-(diethylamino)ethylester (SDZ205557) and the benzimidazolone derivative, endo-6-methoxy-8-methyl-8-azabicyclo[3.2.1]oct3-yl-2,3-dihydro-2-oxo-1H-benzimidazole-1carboxylate hydrochloride (DAU6285) (Fig.1.10) are more potent and selective antagonists for 5-HT$_4$ receptors in comparison with ICS205-930 (Buchheit et al., 1991; Dumuis et al., 1992). In 1993, Grossman et al. developed a radioligand for 5-HT$_4$ receptors using the indole derivative, [1-[2-[(methylsulphonyl-amino)ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate (GR113808) (Fig.1.10), a highly potent and selective 5-HT$_4$ antagonist. Specific [^{3}H]GR113808 binding in homogenates of guinea-pig striatum and hippocampus is potently and stereoselectively inhibited by agonists and antagonists acting at the 5-HT$_4$ receptor but not by compounds selective for other 5-HT receptors or other neurotransmitter receptors. Autoradiographic studies using [^{3}H]GR113808 in guinea-pig and rat brain showed a discrete localisation with high concentration of binding in brain areas such as striatum, substantia nigra and olfactory tubercle.

1.3.5 Other 5-HT Receptors

Genes encoding two putative 5-HT receptors, 5-HT$_{5A}$ and 5-HT$_{5B}$, have been isolated from mouse and rat cDNA libraries

(Plassat et al., 1992; Erlander et al., 1993; Matthes et al., 1993). The proteins encoded by these genes possess all the features of a seven transmembranes, G-protein coupled receptor, but no second messenger system has yet been identified. Experiments hybridising 5-HT$_{5A}$ mRNA show its localisation in the cortex, hippocampus and hypothalamus. 5-HT$_{5B}$ receptor mRNA has been found only in the hàbenula and CA1 field of the hippocampus. A 5-HT$_6$ receptor gene has been recently cloned from a rat cDNA library (436 amino acids) (Monsma et al., 1993; Ruat et al., 1993a). This receptor exhibits poor structural homology with other 5-HT receptors (<40%). Although it is positively linked to cAMP formation and in this respect resembles the 5-HT$_4$ receptor, the pharmacological profiles of these two receptors are very different. In situ hybridization studies indicate a concentration of the 5-HT$_6$ receptor message in the striatum, olfactory tubercle, cortex and hippocampus. The 5-HT$_7$ receptor is the most recent receptor to have been characterised, the gene having been cloned from rat (448 amino acids) and mouse (448 amino acids) cDNA libraries (Ruat et al., 1993b; Plassat et al., 1993). This receptor couples to the activation of adenylate cyclase like the 5-HT$_4$ and 5-HT$_6$ receptors. In brain, mRNA is particularly well represented in the thalamus, hippocampus and discrete regions of the limbic system.

1.3.6 5-HT Transporter

5-HT is concentrated in neurons by two transport processes. One is a re-uptake process which acts to terminate the action of 5-HT released from presynaptic nerve terminals,

and the other is part of the storage process in the synaptic vesicles (Kannengiesser at al.,1973). The neuronal plasma membrane transporter for 5-HT is functionally dependent on the transmembranal electrochemical gradients generated by the ouabain sensitive Na^+/K^+ ATPase (Tissari et al., 1969; Bogdanski et al., 1979). Many studies on the plasma membrane 5-HT transport have been carried out using platelets because blood platelets are considered to accumulate and store 5-HT in a manner similar to that occurring in serotonergic nerve terminals (Pletscher, 1968; Stahl, 1977). In particular, the use of plasma membrane vesicles prepared from platelets has contributed towards the understanding of the mechanism of 5-HT transport since experimental work can be carried out with this preparation in the absence of intracellular events such as storage and metabolic enzymes. Studies with platelet plasma membrane vesicles provided direct evidence that 5-HT is accumulated only when appropriate Na^+/K^+ gradients are constructed (Rudnick, 1977; Talvenheimo et al., 1979). Accumulation of 5-HT by platelet plasma membrane vesicles under physiological conditions (high internal K^+, low internal Na^+) suggests that 5-HT is actively transported into intact platelets prior to uptake into storage organelles. In this system, ouabain does not inhibit 5-HT transport, indicating that 5-HT uptake is not directly coupled to the Na^+/K^+ ATPase. On the other hand, uptake is inhibited strongly by tricyclic antidepressants and by ionophores such as gramicidin which catalyze transmembrane exchange of Na^+ and K^+ dissipating the transmembrane ionic gradients (Rudnick, 1977). In addition, 5-HT transport is absolutely dependent on the presence of

external Na^+ and Cl^- (Rudnick, 1977; Nelson and Rudnick, 1982). From these studies using platelet plasma membrane vesicles, a model for the translocation cycle of the 5-HT transporter was proposed (Rudnick, 1977; Rudnick and Nelson, 1978; Nelson and Rudnick, 1982). In the first step of the cycle 5-HT, Na^+ and Cl^- bind to the transporter on the extracellular side and then they are translocated to the cytoplasmic surface where they dissociate. K^+ binds to the cytoplasmic side of the transporter and is translocated to the exterior side and the cycle is completed when K^+ dissociates (Fig.1.11). Studies with plasma membrane vesicles from mouse brain showed that the pharmacology of the 5-HT transporter corresponds well with that reported for rat brain synaptosomes (O'Reilly and Reith, 1988). Amine uptake in brain vesicles is driven by a Na^+ gradient ($[Na^+]out > [Na^+]in$) and a K^+ gradient ($[K^+]in > [K^+]out$). External Na^+ cannot be replaced by Li^+, K^+ or sucrose, and external Cl^- gives higher uptake than NO_2^- or $S_2O_3^{2-}$. In addition, internal K^+ cannot be replaced by Li^+ or sucrose. Thus the mechanism for 5-HT uptake proposed by studies using plasma membrane vesicles from mouse brain is similar to that for the corresponding amine transporter in platelets (O'Reilly and Reith, 1988; Reith et al., 1989).

The uptake of 5-HT into the storage vesicle occurs by the operation of two sequential processes (Toll and Howard, 1978; Johnson and Scarpa, 1979). First, a vacuolar ATP-driven H^+ pump generates an electrochemical gradient across the vesicular membrane for H^+ (inside > outside). The subsequent coupling of protons to the 5-HT transporter then provides the energy for transport of the biogenic amine with the driving force coming

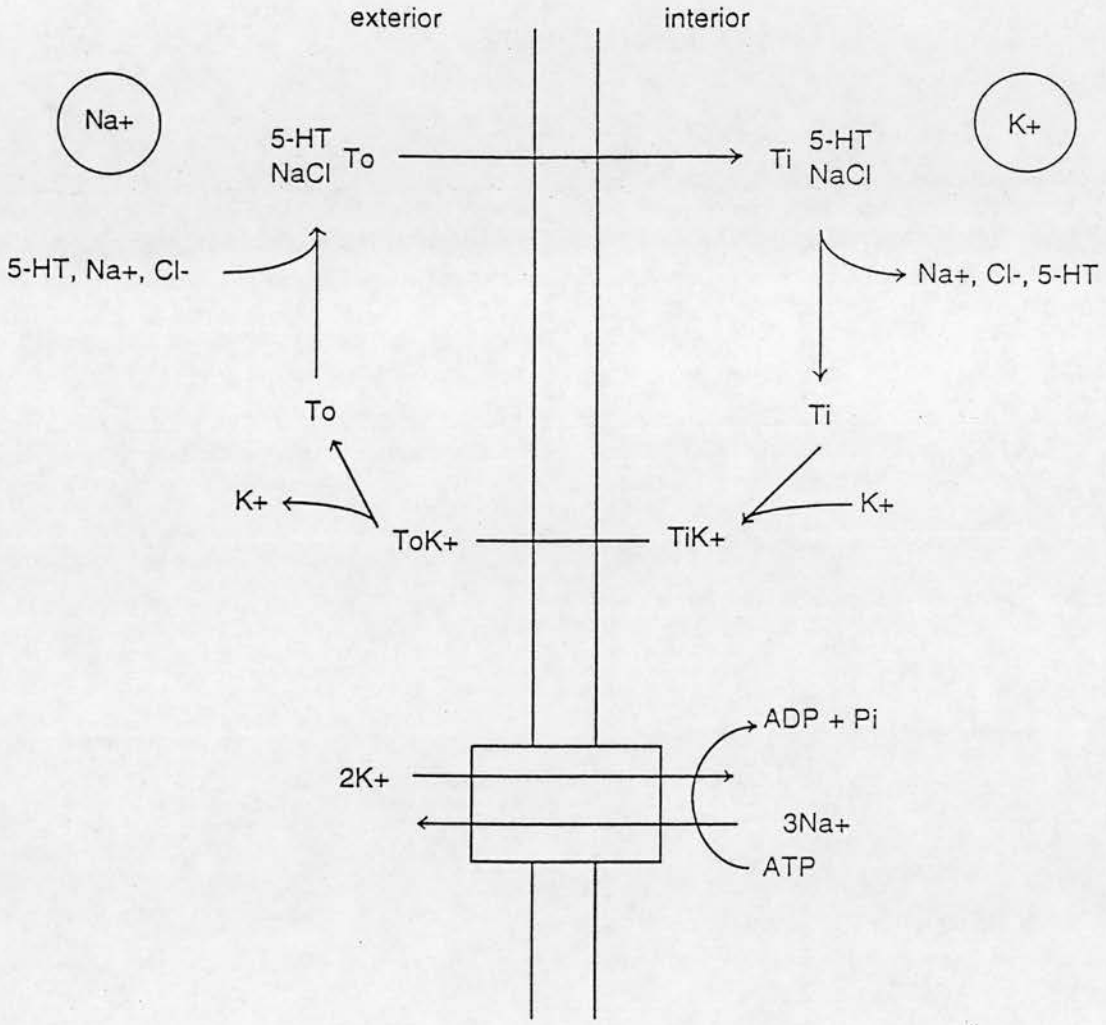
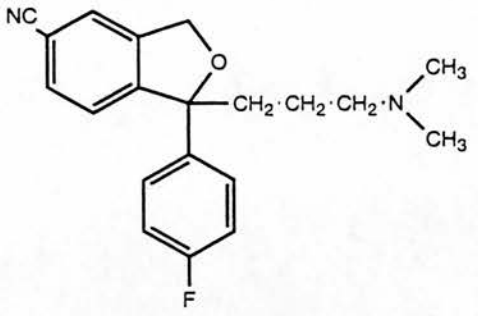


Fig. 1.11 Model for translocation cycle of the 5-HT transporter. In the first step of 5-HT uptake in the platelets, 5-HT, Na+ and Cl- bind to the transporter (To) on its exterior plasma membrane side and then they are translocated across the membrane. Upon dissociation of this complex at the cytoplasmic side of the membrane, the binding of K+ to the transporter (Ti) serves to reorientate the 5-HT binding site to the extracellular membrane surface. (adapted from Graham and Langer, 1990)

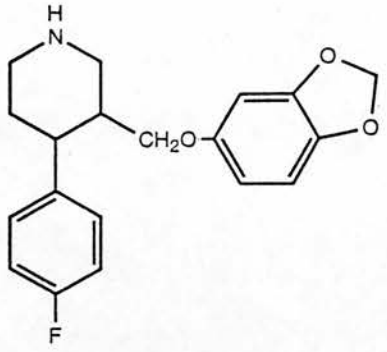
from the movement of H+ ions down their electrochemical potential gradient. Therefore this transporter functions in an antiport configuration as 5-HT is taken up into the storage vesicle in exchange for H+ ion extrusion. 5-HT accumulation is dependent on both a transmembrane pH difference (ΔpH) and a transmembrane electrical potential difference ($\Delta\Psi$) (Rudnick and Clark, 1993). Because the amine substrate is exchanged for the equivalent of two H+ ions, a 10-fold H+ concentration gradient (one pH unit) will lead to a 100-fold gradient of substrate. The vesicular monoaminergic uptake system is inhibited by reserpine which binds at the site of amine recognition with a Ki value in the subnanomolar range (Scherman and Henry, 1984; Darchen et al., 1989). The vesicle transport is relatively non-selective and appears to be the same in all amine storage organelles.

1.3.7 5-HT Uptake Inhibitors

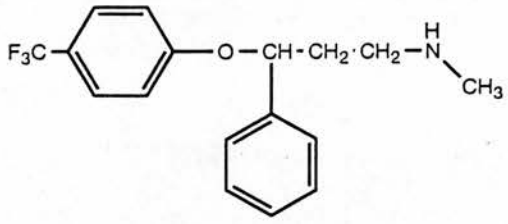
Inhibition of amine uptake into serotonergic and noradrenergic terminals is a pharmacological characteristic of the tricyclic antidepressants such as imipramine and chlorimipramine (Fig.1.12) (Kannengiesser et al., 1973; Richelson and Pfenning, 1984). In addition, a deficit in serotonergic neurotransmission has been implicated in the aetiology of clinical depression (Goodwin and Post, 1983). Therefore, during the past two decades a number of pharmaceutical companies have developed potent and selective 5-HT uptake inhibitors (SSRI's) as new antidepressant drugs, the most widely used drug being prozac (fluoxetine) (Fig.1.12). Clinical studies with SSRI's such as fluoxetine and citalopram



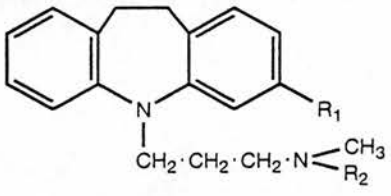
Citalopram



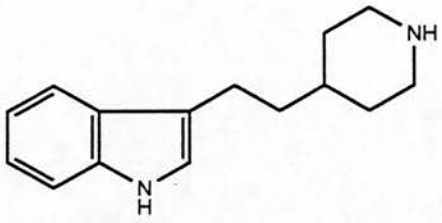
Paroxetine



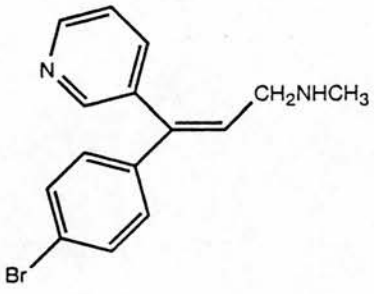
Fluoxetine



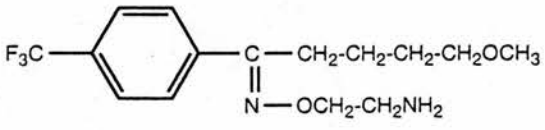
Imipramine : R_1=H, R_2=CH_3
Chlorimipramine : R_1=Cl, R_2=CH_3
Desipramine : R_1=H, R_2=H



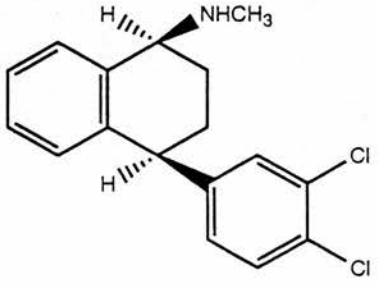
Indalpine



Norzimelidine



Fluvoxamine



Sertraline

Fig. 1.12 5-HT uptake inhibitors

(Fig.1.12) have revealed that they are as effective as the tricyclic antidepressants and are devoid of the undesirable side-effects such as nausia, dry mouth and cardiac toxicity seen with tricyclic antidepressant treatment (Pedersen et al.,1982; Benfield et al., 1986; Milne and Goa, 1991). Other possible applications for SSRI's include their use in the treatment of obesity and alcoholism. Preliminary studies have indicated that fluoxetine leads to reductions in body weight in nondepressed obese human patients (Ferguson, 1986). In addition, citalopram has been shown to decrease alcohol consumption in nondepressed heavy drinkers (Naranjo et al., 1987), and it is interesting to note that fluoxetine has been claimed to enhance memory processing in certain animal models (Flood and Cherkin, 1987).

In initial studies [^{3}H]imipramine (Fig.1.12) was used to label the 5-HT transporter (Langer et al., 1980a, 1980b). Recently more selective 5-HT uptake inhibitors such as [^{3}H]citalopram (D'Amato et al., 1987a), [^{3}H]paroxetine (Habert et al., 1985), [^{3}H]norzimelidine (Hall et al., 1982) and [^{3}H]indalpine (Benavides et al., 1985) were utilised to label 5-HT uptake sites in brain (Fig.1.12). In autoradiographic studies, the distribution patterns of [^{3}H]paroxetine and [^{3}H]citalopram binding in rat brain are consistent with the organisation of serotonergic terminals and cell bodies (DeSouza and Kuyatt, 1987; Hrdina et al., 1989; D'Amato et al., 1987a). The effects of various 5-HT uptake inhibitors on transport of tritiated amine (5-HT, noradrenaline (NA), dopamine (DA)) into rat brain synaptosomes were compared by Hyttel (1982). Paroxetine (IC_{50} = 0.31 nM), chlorimipramine (IC_{50} = 1.5 nM)(Fig.1.11), citalopram (IC_{50} = 1.8 nM), indalpine (IC_{50} = 2.4 nM) and fluoxetine (IC_{50} = 6.9 nM) are among the most

potent 5-HT uptake inhibitors. Citalopram is the most selective of the SSRI's showing a much greater ratio (IC_{50} for NA / IC_{50} for 5-HT) than other compounds. Most 5-HT uptake inhibitors are devoid of an inhibitory effect on amine uptake into dopaminergic neurones. Radioligand binding studies showed that paroxetine and citalopram have little affinity for α_1, α_2 or β adrenoceptors, dopamine (D_2), 5-HT_1, 5-HT_2 or histamine (H_1) receptors at concentrations below 1 μM (Thomas et al., 1987). Paroxetine has weak affinity for muscarinic receptors (Ki = 89 nM) whereas citalopram (Ki = 2900 nM) is much less potent. Thus while citalopram is the most selective 5-HT uptake inhibitor, paroxetine is the most potent uptake inhibitor. Administration of paroxetine and citalopram at doses up to 5 mg/kg, p.o. and 10 mg/kg, i.p. respectively produces a dose-related inhibition of [^{3}H]5-HT uptake into rat brain synaptosomes ex vivo with little effect on [^{3}H]NA uptake (Hyttel, 1982; Thomas et al., 1987). In addition, both compounds can prevent the reduction of rat brain 5-HT content by 5-HT depleting drugs such as PCA. These observations indicate 5-HT uptake blockade in vivo by paroxetine and citalopram. It has been demonstrated that [^{3}H]paroxetine and [^{3}H]citalopram are selective agents for in vivo labelling of cerebral 5-HT uptake sites (Scheffel and Hartig, 1989; Scheffel and Ricaurte, 1990; Hume et al., 1991). Therefore paroxetine and citalopram might be potentially useful prototype molecules for the development of SPET ligands for the 5-HT transporter in serotonergic neurones.

It has been proposed that [^{3}H]2-nitroimipramine may be a valuable tool for isolation and subsequent purification of the 5-HT transporter site since this ligand has a higher specific

activity and a higher affinity than [^{3}H]imipramine in human platelets and rat brain membranes, having an extremely slow dissociation rate ($t_{1/2}$ > 30 hr) when incubations are carried out at 0-4°C (Rehavi et al., 1982). Rotman and Pribluda (1982) showed that a photoaffinity ligand for the 5-HT transporter, [^{3}H]azidoimipramine, binds covalently to guinea-pig brain synaptosomes and platelets. Studies with more selective compounds than imipramine, such as paroxetine and citalopram, could provide more suitable photoaffinity ligands for isolating the 5-HT transporter.

The aim of the work presented in chapter 3 was therefore to examine the possibility of using citalopram derivatives as photoaffinity and SPET ligands for the 5-HT transporter.

A reduction of cortical and hippocampal [^{3}H]imipramine binding site densities has been reported in suicide and depression using post-mortem human brain (Stanley et al., 1982; Perry et al., 1983). On the other hand, the binding of [^{3}H]imipramine has been reported to be unchanged in post-mortem human brain from suicide victims with a clinical history of depression (Owen et al., 1986). In addition, it has been reported that there is no significant difference in [^{3}H]paroxetine binding site density in post-mortem human brain from depressed suicides compared with controls (Lawrence et al., 1990). D'Amato et al. (1987b) reported a reduction in [^{3}H]citalopram binding site density in post-mortem human brain with Alzheimer's disease (AD) and Parkinson's disease without a change in the affinity of the ligand for the remaining transporters. Thus [^{3}H]citalopram and [^{3}H]paroxetine appear to

be useful tools for studies of the 5-HT transporter in post-mortem human brain with various CNS disorders. On the other hand, Middlemiss et al. (1986) showed that a loss of [^{3}H]8-OH-DPAT binding sites is observed in frontal cortex from post-mortem AD brain in comparison with normal brain, suggesting that 5-HT$_{1A}$ receptors are reduced in AD as well as 5-HT uptake sites. However in general it is not clear whether these changes of serotonergic neurones are specific since non-specific neuronal loss is observed in neurodegenarative disorders such as AD.

The aim of the work presented in chapter 4 was to examine the neuronal location of 5-HT$_{1A}$ receptors using [^{3}H]8-OH-DPAT and 5-HT uptake sites using [^{3}H]citalopam in rat forebrain following cholinergic and serotonergic lesions.

CHAPTER 2

CHARACTERISATION OF THE NMDA RECEPTOR ANTAGONIST, FR115427

2.1 INTRODUCTION

Non-competitive NMDA antagonists such as PCP, TCP and MK-801 have been used to develop in vitro ligand binding assays that effectively monitor the state of activation of the NMDA receptor linked ion channel. Studies of [^{3}H]MK-801 and [^{3}H]TCP binding to rat brain membranes showed that MK-801 is the most potent inhibitor in comparison with other PCP-like compounds (Wong et al., 1988). PCP and TCP interact with other sites such as the haloperidol-sensitive sigma receptor and dopamine reuptake sites with lower affinity than for the PCP recognition sites in the NMDA receptor ion channels (Wong et al., 1988; Rothman et al., 1989). However, the affinity of MK-801 for the sigma receptor and dopamine reuptake sites is much lower than those of PCP and TCP. Thus MK-801 is the most potent and selective non-competitive NMDA antagonist among a number of the well-known PCP-like compounds. For these reasons, [^{3}H]MK-801 has been most commonly used for studying the NMDA receptor linked ion channels. [^{3}H]MK-801 binding sites in rat brain membranes are heat-labile, stereoselective and regionally specific (Wong et al., 1986). As described in chapter 1, [^{3}H]MK-801 binding is modulated by various compounds such as L-glutamate, glycine, Zn^{2+} and polyamines, acting at distinct recognition sites. Recently, Bakker et al. (1991) reported binding of [^{3}H]MK-801 to a gel filtered (to remove endogenous compounds) rat brain membrane preparation solubilised by sodium deoxycholate. L-Glutamate, glycine, polyamines and desipramine (zinc recognition site) showed effects on soluble NMDA receptors similar to those observed in rat brain membranes, indicating that their sites of action are directly on

the receptor protein. In quantitative autoradiographic binding experiments using rat brain sections, [^{3}H]MK-801 binding was heterogeneously distributed throughout the brain with high concentrations in the hippocampus, cerebral cortex and thalamus, and was consistent with the pattern of NMDA receptors labelled with [^{3}H]L-glutamate and [^{3}H]TCP (Bowery et al., 1988; Sakurai at al., 1991). The pharmacology of [^{3}H]MK-801 binding in rat brain sections was similar to that described in binding studies of the PCP recognition sites using brain membranes. In addition, [^{3}H]MK-801 binding studies using human postmortem brain tissue showed the existence of high and low affinity binding sites (Quarum et al., 1990). The high affinity site showed a different regional distribution of receptor density (cortex > hippocampus > striatum) compared to the low affinity binding site (cerebellum > brainstem). The regional distribution and pharmacology of [^{3}H]MK-801 binding in the human brain were similar to that reported using [^{3}H]MK-801 in rat brain (Bowery et al., 1988; Sakurai at al., 1991).

Price et al. (1988a,b) reported the characterisation of in vivo receptor binding with [^{3}H]MK-801 in mouse brain. In vivo binding of [^{3}H]MK-801 was inhibited by various non-competitive NMDA antagonists such as MK-801 and TCP. The potency of these drugs was highly correlated with their functional effects as antagonists of NMDA-induced convulsions. Recently it has been demonstrated that (+)-3-[^{125}I]iodo-MK-801 is a useful radioligand for in vitro and in vivo autoradiography and that receptor-mediated localisation of a similar MK-801 analogue may allow in vivo imaging of the NMDA receptor complex (Gibson et al., 1992). In vivo localisation of (+)-3-[^{125}I]iodo-MK-801 in

rat brain is stereoselective and blocked by coinjection of unlabelled MK-801 (4 mg/kg) and is in concordance with in vitro autoradiographic studies.

It has been shown that an NMDA receptor antagonist, an isoquinoline derivative FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride, (+)FR(HCl salt)) (Fig.2.1) prevents hippocampal damage in a gerbil global cerebral ischaemia model (Nakanishi et al., 1994) and reduces cerebral infarct volume and neurological deficit in a rat MCA occlusion model (Katsuta et al., 1995), indicating that (+)FR might be useful in the treatment of human ischaemic neuropathologies such as stroke and trauma. However, the mode of action of (+)FR at the NMDA receptor has not been defined.

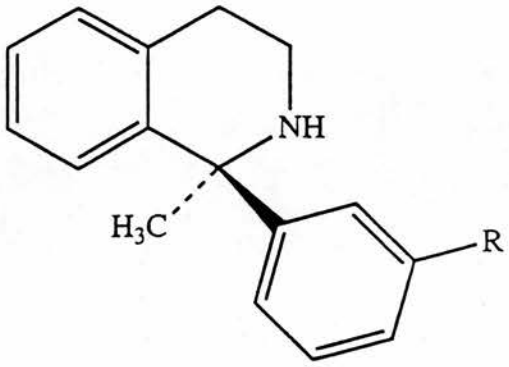
The purpose of the work presented in this chapter was to examine the mode of action of (+)FR using ligand binding techniques. Initially the effects of (+)FR and its analogues on the binding of [^{3}H]MK-801 to rat cortical membranes, including the modulatory effect of L-glutamate, were examined and compared with MK-801. Secondly, tritium-labelled (+)FR was synthesised and a method developed for purifying millicurie quantities of [^{3}H](+)FR. Subsequently its in vivo distribution in rats, following intravenous injection, was studied.

2.2 METHODS

2.2.1 Preparation of Rat Brain Membranes

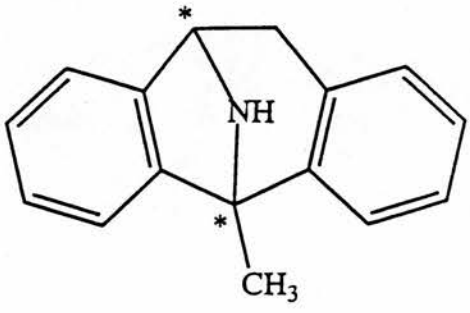
a) Whole brain membranes

The cerebral cortex from male Sprague Dawley rats (200-300g) was dissected and homogenised in 40 vols (V/W) of



R = H : (+)-1-methyl-1-phenyl-1,2,3,4-tetrahydro-isoquinoline hydrochloride; FR115427

R = Cl : (+)Cl-FR



MK-801 (Dizocilpine)

Fig. 2.1 Chemical structures of FR115427 analogues and MK-801

ice-cold 5 mM Tris-HCl buffer (pH 7.4 at 25°C) using a glass teflon homogeniser. The homogenate was centrifuged at 50,000g at 4°C for 10 min. To remove endogenous excitatory amino acids the resultant pellet was resuspended in 40 vols of Tris-HCl buffer, incubated at 37°C for 30 min then recentrifuged (50,000g, 4°C, 10 min). After the second wash, the pellet was resuspended with 10 vols of Tris-HCl buffer and stored at -20°C in aliquots sufficient for individual experiments until required. On the day of the experiment, the membrane suspension was thawed at room temperature, diluted to 40 vols with Tris-HCl buffer and centrifuged (50,000g, 4°C, 10 min). Finally, the pellet was resuspended with 50 vols of 5 mM Tris-HCl buffer (pH 7.4 at 25°C) and kept at 4°C until required for the binding assay.

b) Synaptosomal membranes

The cerebral cortex from male Sprague Dawley rats (200-300 g) was dissected and homogenised in 15 vols (V/W) of ice-cold 0.32 M sucrose using a glass teflon homogeniser. The homogenate was centrifuged at 1,000g at 4°C for 10 min, then the supernatant centrifuged at 17,000g at 4°C for 20 min. To remove endogenous excitatory amino acids the resultant P_2 pellet was lysed with 40 vols of glass distilled water (GDW), incubated at 37°C for 30 min then centrifuged (50,000g, 4°C, 10 min). The supernatant was discarded and the pellet washed by resuspension in GDW (40 vols) and centrifugation (50,000g, 4°C, 10 min). The resultant pellet was resuspended with 10 vols of GDW and stored at -20°C until required. On the day of the experiment, the membrane suspension was thawed at room

temperature, diluted to 40 vols with GDW and centrifuged (50,000g, 4°C, 10 min). The final pellet was resuspended in 30 vols of 5 mM Tris-HCl buffer (pH 7.4 at 25°C) and kept at 4°C until required for the binding assay.

2.2.2 [^{3}H]MK801 Binding to Rat Cortical Membranes

[^{3}H]MK-801 binding to rat cortical membranes was carried out according to the method of Wong et al. (1988). Duplicate 0.5 ml samples of membrane suspension were preincubated with 10 concentration of test drug (10^{-10} M to 10^{-4} M, final concentrations) or 5 mM Tris-HCl buffer, pH 7.4, (total binding) for 2 min at 25°C before addition of [^{3}H]MK-801 (specific activity 28.8 Ci/mmol, final concentration 1 nM) to give a final assay volume of 1 ml. Samples were vortexed briefly then incubated at 25°C for 45 min to reach equilibrium binding. Incubation was terminated at room temperature by rapid filtration under vacuum on a Brandel Cell Harvester using Whatman GF/B filters then washed twice with 5 ml of 5 mM Tris-HCl buffer. The filters were transferred to scintillation vials and 100 µl of formic acid (100%) added to digest the membrane protein followed 10 min later by 4 ml of scintillation fluid (Packard Emulsifier Safe). The radioactivity in the vials was measured in a Packard 1900CA liquid scintillation analyser using automatic quench correction. Unlabelled (+)MK-801 (10 µM final concentration) was used to define specific binding. Assays were carried out in the absence or presence of added L-glutamate (final concentration 10 µM). Quadruplicate 100 µl samples of [^{3}H]MK-801 as standards were counted in order for the exact ligand concentration to be calculated for individual

experiments.

2.2.3 Data Analysis of Binding Experiments

Data from individual experiments were analysed by least squares fit to the logistic expression :

$$Y = MX^P / (X^P + IC_{50})$$

where Y is the amount bound at the inhibitor concentration, X ; P corresponds to the Hill co-efficient, IC_{50} is the inhibitor concentration giving 50% inhibition of [^{3}H]MK-801 binding. Equilibrium dissociation constants (Ki values) for inhibitors of binding were calculated using the equation (Cheng and Prusoff, 1973) :

$$Ki = IC_{50} / (1 + L/K_D)$$

where L is the [^{3}H]MK-801 ligand concentration used in the experiment and K_D is the equilibrium dissociation constant for MK-801. When the inhibitor used was unlabelled (+)MK-801, its K_D value was calculated using the equation :

$$K_D = IC_{50} - L$$

and subsequently the density of [^{3}H]MK-801 binding sites calculated from the equation :

$$b = Bmax\ L / (L + K_D)$$

Where b is the amount of [^{3}H]MK-801 bound (fmol/mg tissue) at the [^{3}H]MK-801 ligand concentration L. The values for K_D, Ki and Bmax were expressed as mean ± S.E.M. from three to six independent experiments. The Student's t-test or Dunnett's t-test was used for statistical comparison between drugs or conditions when necessary.

2.2.4 Preparation and Purification of [^{3}H]FR115427

a) Column Chromatography of (+)FR and (+)Cl-FR

Since the amounts of [^{3}H](+)FR required to be purified exceeded the capacity of our HPLC, an open column chromatography method was used to isolate, purify and identify FR115427 and in particular to separate it from the precursor used for the tritiation, (+)-1-(3-chlorophenyl)-1-methyl-1,2,3,4-tetrahydroisoquinoline ((+)Cl-FR, Fig.2.1). Waters Preparative C-18 resin (55-105μ, 1.5g) in 20ml of solvent (CH_3CN : aq. 0.1% CF_3COOH (TFA); 3:7) was sonicated for 3 min to remove air bubbles. The resin was applied slowly to a disposable plastic column (BioRad, 0.7 x 8 cm) until a packed 5 cm column of resin was obtained. A sample of solvent (200 μl) containing 200 μg (+)FR(free base) and 200 μg (+)Cl-FR(free base) was then carefully applied to the column. The column was eluted with solvent (CH_3CN : aq. 0.1% TFA ; 3:7) and 350 μl fractions collected in microcentrifuge tubes. Each fraction was frozen in dry ice (-80°C) and the solvent was removed by freeze drying. After addition of GDW (20 μl) to each fraction and vortexing, a 5 μl was applied to a silica gel plate and thin layer chromatography (TLC) carried out using $CHCl_3$: MeOH = 9:1 as developing solvent. (+)FR and (+)Cl-FR were detected under U.V. light (254 nm) and R_F values calculated.

b) Synthesis and Purification of [^{3}H]FR115427

(+)Cl-FR (23.2 mg in a preweighed ampoule), the mono-chlorinated aromatic precursor of (+)FR (Fig.2.1) was custom tritiated by Amersham International. (+)Cl-FR in

methanol was tritiated for 2 hr at room temperature with tritium gas. After removal of methanol, [^{3}H](+)FR was extracted with ethyl acetate and water, and stored at a radiochemical concentration of 10 mCi/ml in ethyl acetate. A batch of the crude material (200 mCi) was delivered to the Department of Pharmacology, University of Edinburgh and stored in 0.5 ml aliquots (10 mCi/ml) under liquid nitrogen prior to purification.

For purification of [^{3}H](+)FR, after removal of ethyl acetate, 4.02 mCi of crude [^{3}H](+)FR was dissolved with 402 µl of the eluting solvent (CH_3CN : aq. 0.1% TFA; 3:7) then 4 mCi (400 µl) applied to the column. The open column method was carried out as described above in order to isolate authentic [^{3}H](+)FR. Fractions (500 µl) were collected in microcentrifuge tubes and a subsample (2 µl) of each fraction was diluted 1 : 10,000 with GDW and recovery determined by scintillation counting. In order to calculate the exact amount of crude [^{3}H](+)FR added to the column, standards were obtained by diluting 2 µl of the original 402 µl of radioactivity 1 : 12,500 and counting 100 µl in quadruplicate. Fractions corresponding to [^{3}H](+)FR were combined and freeze-dried. Purified [^{3}H](+)FR, now as the trifluoroacetate salt (TFA salt), was diluted to 10 µM in GDW and stored under liquid nitrogen in volumes sufficient for individual experiments. The specific activity (15.7 Ci/mmol) was calculated from Amersham's report that 1.24 Ci was obtained from catalytic reaction of 23.2 mg of (+)Cl-FR (M.W. 294.22) with tritium gas. Thus the concentration of [^{3}H](+)FR was calculated from the amount of radioactivity of [^{3}H](+)FR.

2.2.5 Purity of [^{3}H]FR115427

The purity of [^{3}H](+)FR was checked by TLC. Each 3 or 2 µl of [^{3}H](+)FR (1.78 µCi/ml) and 3 or 2 µl respectively 10 mM unlabelled (+)FR as carrier was spotted on silica gel plates. After TLC development by $CHCl_3$-MeOH (9:1), the plate was cut into 3 mm by 10 mm strips parallel to the origin. Each strip was transferred to a scintillation vial and 300 µl glass distilled water was added to elute the radioactivity. After 1 hr, 4 ml scintillation fluid added and the radioactivity in each strip determined by liquid scintillation counting. The radioactivity in each strip was calculated as a recovery % from the total radioactivity applied to the origin. Immediately before cutting the TLC, (+)FR was detected under U.V. light, its Rf value and the strip numbers corresponding to the spot noted.

2.2.6 In Vivo Distribution of [^{3}H]FR115427

Male Sprague Dawley rats (250-320 g) were allowed access to food and water ad libitum prior to the experiments. Under urethane anaesthesia (10% solution, 10 ml/kg, i.p.), the left femoral vein was catheterised with polyethylene tubing for drug administration. Rats were injected over 30 sec with 500 µl of saline containing 50 µCi of [^{3}H](+)FR (corresponding to 3.2 nmol of [^{3}H](+)FR) and 10 unit of heparin through the cannula placed in the left femoral vein. To complete the drug injection, 500 µl of heparinised saline was used to flush the cannula immediately following infusion of the [^{3}H](+)FR. At various times post-injection (15, 30, 60, 90 and 180 min), samples of blood (~5ml) were taken in a heparinised syringe from the inferior vena cava then the rats were killed by decapitation.

Three animals were used for each time point. The brains were removed, divided into 7 regions : rostral cortex, caudal cortex (cerebral cortex - rostral cortex), hippocampus, striatum, brainstem, midbrain and cerebellum. In addition, liver, kidney and heart were removed, blotted dry and weighed. Samples, 120 to 400 mg, of each organ were taken, again blotted to remove as much blood or urine as possible and transferred to glass scintillation vials, then treated the same way as brain tissue for determination of radioactivity. The tissues were weighed in glass scintillation vials and dissolved with tissue solubiliser (Soluene-350, 1ml/200mg) at 37°C for 2 days. After being cooled to room temperature, 10 ml of Packard Hionic-Fluor scintillation fluid was added to each sample and radioactivity determined in a Packard 1900CA liquid scintillation analyser. For the 60 min and 180 min rats, the right femoral artery was also cannulated to obtain blood samples at several times post injection of [^{3}H](+)FR. At each time the first 100 µl of blood was discarded since it probably represented the dead volume in the cannula. A 300 µl sample was then collected in a heparinised microcentrifuge tube. A 200 µl sample was transferred to a glass scintillation vials, and 1ml of Soluene-350 was added and left for 2 hr. 0.5 ml of 30% hydrogen peroxide (H_2O_2) was then added to bleach the blood and left for 1hr. After incubation of vials at 40°C for 30 min, 10 ml of Packard Hionic-Fluor was added and radioactivity determined. The same procedure was carried out for the 5 ml blood samples taken from the vena cava of all animals. The results for the tissue distribution study were expressed as % injected dose per gram of tissue (%D/g) or fmol per milligram of

tissue (fmol/mg), and results for the blood distribution study were expressed as % injected dose per ml of blood (%D/ml). In order to calculate the exact amount of [^{3}H](+)FR injected to rats, standards were obtained by diluting radioactivity used for injection 1 : 200 and counting 100 µl in quadruplicate. The data for whole brain were obtained from summation of the amounts of radioactivity and weight of all brain regions.

2.2.7 Pharmacokinetic Analysis

The data for radioactivity in blood expressed as %D/ml after [^{3}H](+)FR injection were fitted to biexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-\alpha t)} + B \times e^{(-\beta t)}$, where t is time after administration, Y is the measured radioactivity, A and B are the coefficients of the exponential terms, α is the apparent distribution rate constant, β is the apparent terminal rate constant. The data for radioactivity in brain and peripheral tissues were fitted to monoexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-Ke \cdot t)}$, where Ke is the elimination rate constant. The following pharmacokinetic parameters were calculated : 1) half-life ($t_{1/2}$) = 0.693/Ke or α or β; 2) the area under the radioactivity concentration-time curve from zero to time infinity (AUC). AUC was calculated using the trapezoid method (Chiou, 1978).

2.2.8 Materials

FR compounds and MK-801 were synthesised by Mr.M.Ohkubo in the New Drug Research Laboratories, Fujisawa Pharmaceutical

Co. Ltd., Osaka, Japan. FR compounds obtained as HCl salts were dissolved in GDW, FR compounds obtained as free bases were dissolved in equimolar HCl, and MK-801 was dissolved in aqueous diluted HCl. [^{3}H](+)MK-801 (specific activity : 28.8 Ci/mmol) was obtained from NEN. [^{3}H](+)MK-801 was diluted to 1 μM in GDW and stored under liquid nitrogen in volumes sufficient for individual experiments. Normally between 10 and 16 sets of tubes (24 tubes/set) were processed on the Brandel Cell Harvester in a single experiment.

2.3 RESULTS

2.3.1 [^{3}H]MK-801 Binding to Rat Cortical Whole Membranes

In order to examine the interaction of (+)FR with all NMDA receptors including potentially non-neuronal and synaptic NMDA receptors, the two preparations (whole and synaptosomal membranes) were used for [^{3}H]MK-801 binding.

The effects of (+)FR, (+)MK-801 and their enantiomers on [^{3}H]MK-801 binding to rat cortical whole membranes were examined in the absence and presence of added L-glutamate (final concentration 10 μM) (Table 2.1). Fig.2.2 shows representive inhibition curves comparing MK-801 and (+)FR as inhibitors of [^{3}H]MK-801 in the presence and absence of added L-glutamate. (+)FR inhibited the binding of [^{3}H]MK-801 to whole membranes with Ki values of 55.2 ± 2.9 nM and 48.1 ± 5.9 nM in the absence and presence of added L-glutamate, respectively. (+)MK-801 was found to be 16-fold more potent than (+)FR as an inhibitor of [^{3}H]MK-801 binding with a K_D value of 3.4 ± 0.2 nM ($p<0.05$ vs (+)FR) in the presence of added L-glutamate. However the difference in affinity between (+)MK-801 and (+)FR

TABLE 2.1 : Inhibition of [^{3}H]MK-801 Binding to Rat Cortical Membranes by Stereoisomers of MK-801 and FR

	K_D / Ki (nM)			
	Whole membranes		Synaptosomal membranes	
	No addition	L-Glutamate	No addition	L-Glutamate
(+)MK-801	7.3 ± 0.8	3.4 ± 0.2**	25.0 ± 6.6#	3.3 ± 0.6*
(-)MK-801	28.3 ± 4.9	15.3 ± 1.6	33.6 ± 8.3	13.9 ± 1.6
(±)MK-801	14.1 ± 0.9	6.9 ± 1.1	30.6 ± 6.3	6.8 ± 1.0
(+)FR	55.2 ± 2.9	48.1 ± 5.9	114.5 ± 30.8	40.0 ± 2.1
(-)FR	4000 ± 269	5270 ± 205	5650 ± 1950	3910 ± 726
(±)FR	121.2 ± 18.0	91.5 ± 6.2	114.7 ± 8.2	64.8 ± 8.0
Bmax (fmol/mg tissue)	57.2 ± 5.4	74.3 ± 8.2	45.7 ± 3.1	48.8 ± 5.9

The affinity of the stereoisomers of MK-801 and FR for [^{3}H]MK-801 binding sites on rat cortical whole membranes or synaptosomal membranes was studied in the absence or presence of added L-glutamate (10 μM) in the assay buffer. Membranes were incubated with [^{3}H]MK-801 (1 nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Ki values were calculated from the equation $Ki = IC_{50} / (1 + L/K_D)$. Values are mean ± S.E.M. of at least three independent experiments. *, ** ; $p<0.05$, 0.01 vs K_D values of (+)MK-801 in the absence of added L-glutamate, # ; $p<0.1$ vs K_D value of (+)MK-801 in the absence of added L-glutamate with whole membranes.

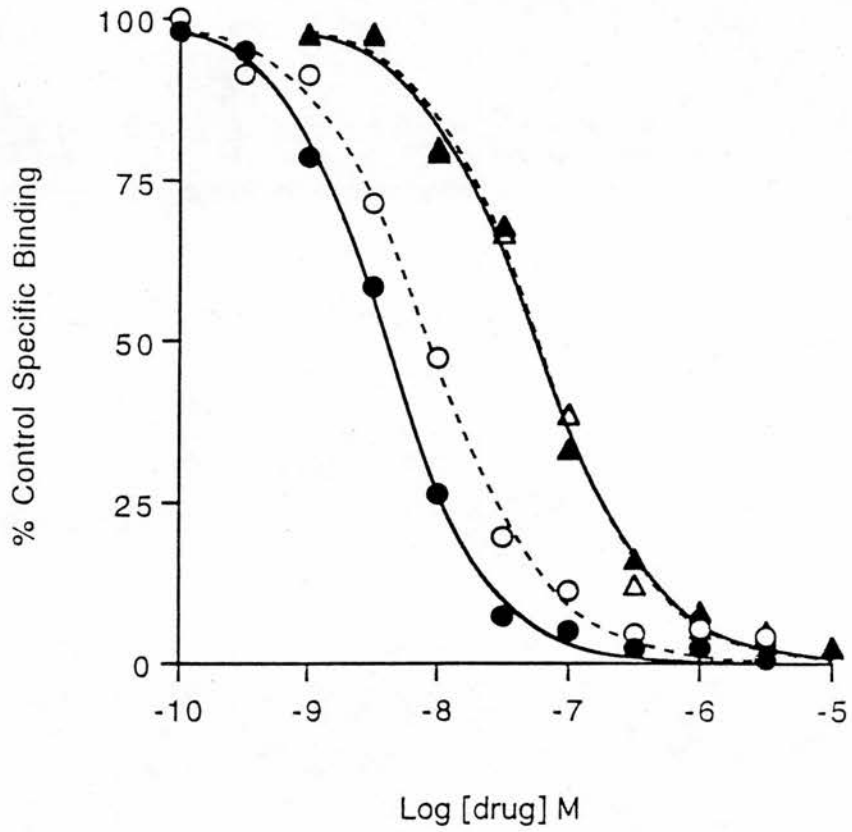


Fig. 2.2 Inhibition of [^{3}H]MK-801 binding to whole membranes by (+) MK-801 and (+)FR : effect of L-glutamate. Cortical whole membranes were incubated with [^{3}H]MK-801 (1nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Assays were carried out in the absence and presence of added L-glutamate (10 μM). Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● (+)MK-801 in the presence of added L-glutamate; ○ (+)MK-801 in the absence of added L-glutamate; ▲ (+)FR in the presence of added L-glutamate; △ (+)FR in the absence of added L-glutamate.

was less apparent in the absence of added L-glutamate ; a K_D value of 7.3 ± 0.8 nM ($p<0.001$ vs (+)FR) and a Ki value of 55.2 ± 2.9 nM were obtained for (+)MK-801 and (+)FR respectively demonstrating a 8-fold difference in affinity. Inhibition of [^{3}H]MK-801 binding by FR and MK-801 showed stereoselectivity but this was far more pronounced in the case of FR. The (-)enantiomers of FR and MK-801 were respectively 110- and 4.5-fold less potent than the corresponding (+)enantiomer in the presence of added L-glutamate. [^{3}H]MK-801 binding was modulated by addition of L-glutamate in the assay buffer. The specific binding of [^{3}H]MK-801 at 1 nM in the presence of added L-glutamate was 2.9 times greater than that in the absence of added L-glutamate (data not shown). The affinity of (+)MK-801 was significantly ($p<0.01$) increased by about 2-fold from 7.3 to 3.4 nM in the presence of added L-glutamate with no significant ($p=0.1122$) effect on Bmax values (57.2 ± 5.4 fmol/mg tissue in the absence of added L-glutamate, 74.3 ± 8.2 fmol/mg tissue in the presence of added L-glutamate). These results suggest that the effect of L-glutamate is to increase the affinity of MK-801, but that there is no change in the population of sites labelled by the ligand. In contrast, the presence of added L-glutamate had little effect on the affinity of (+)FR for [^{3}H]MK-801 binding sites ($p=0.3409$ vs Ki values in the absence of added L-glutamate) (Fig.2.2). A similar result was found for the (-)enantiomers of MK-801 and FR; (-)MK-801 affinity increased about two fold in the presence of added L-glutamate but little effect on the affinity of (-)FR was observed.

2.3.2 [^{3}H]MK-801 Binding to Rat Cortical Synaptosomal Membranes

The effects of (+)FR, (+)MK-801 and their enantiomers on [^{3}H]MK-801 binding to rat cortical synaptosomal membranes in the absence and presence of added L-glutamate are shown in table 2.1. In the presence of added L-glutamate, (+)FR inhibited the binding of [^{3}H]MK-801 to rat cortical synaptosomal membranes with a Ki value of 40.0 ± 2.1 nM ($p<0.001$ vs (+)MK-801) which was 12 times less potent than (+)MK-801 (Ki = 3.3 ± 0.6 nM) (Fig.2.3). On the other hand, (+)FR (Ki = 114.5 ± 30.8 nM, $p<0.05$ vs (+)MK-801) was only 4.5-fold less potent than (+)MK-801 (Ki = 25.0 ± 6.6 nM) in the absence of added L-glutamate. Thus compared with whole membranes, the difference in affinity between (+)MK-801 and (+)FR was less apparent in the absence of added L-glutamate. However there was no significant defference in the affinity of (+)MK-801 and (+)FR between synaptosomal and whole membranes in the absence of added L-glutamate ($p=0.0563$ for (+)MK-801; $p=0.1953$ for (+)FR).

In the presence of added L-glutamate, the affinity of (+)MK-801 for [^{3}H]MK-801 binding sites was significantly increased ($p<0.05$ vs no addition of L-glutamate) by 7.6-fold from 25.0 to 3.3 nM whereas there was no significant change ($p=0.1372$) in the affinity of (+)FR (Fig.2.4). As with whole membranes, inhibition of [^{3}H]MK-801 binding by FR and MK-801 demonstrated stereoselectivity which was more pronounced in the case of FR (Fig.2.3). The (-)enantiomers of FR and MK-801 were respectively 98- and 4.2-fold less potent than the

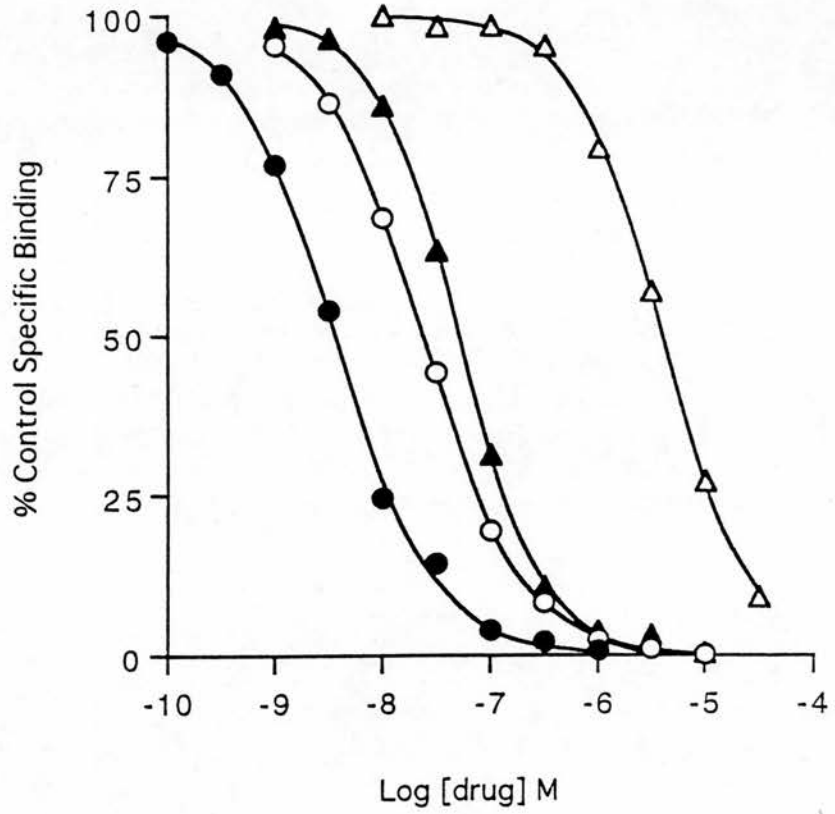


Fig. 2.3 Inhibition of [^{3}H]MK-801 binding to synaptosomal membranes by the (+) and (-)enantiomers of MK-801 and FR. Cortical synaptosomal membranes were incubated with [^{3}H]MK-801 (1nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Assays were carried out in the presence of added L-glutamate (10 μM). Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ●(+)MK-801; ○(-)MK-801; ▲(+)FR; △ (-)FR.

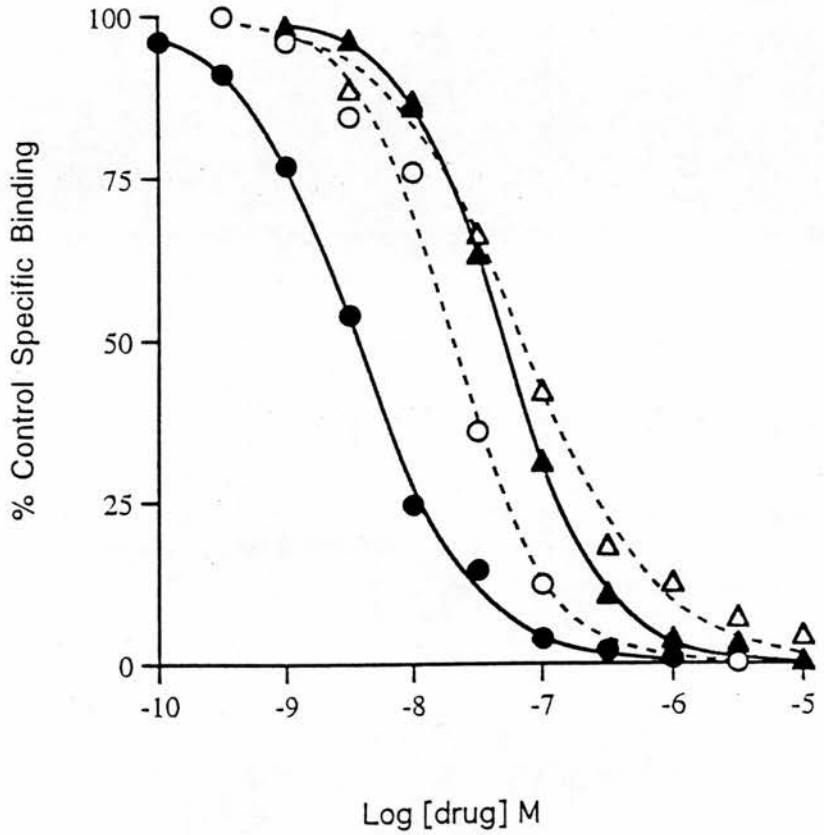


Fig. 2.4 Inhibition of [^{3}H]MK-801 binding to synaptosomal membranes by (+) MK-801 and (+)FR : effect of L-glutamate. Cortical synaptosomal membranes were incubated with [^{3}H]MK-801 (1nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Assays were carried out in the absence and presence of added L-glutamate (10 μM). Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● (+)MK-801 in the presence of added L-glutamate; ○ (+)MK-801 in the absence of added L-glutamate; ▲ (+)FR in the presence of added L-glutamate; △ (+)FR in the absence of added L-glutamate.

corresponding (+)enantiomer in the presence of added L-glutamate. In the presence of added L-glutamate, (+)MK-801 (K_D = 3.3 nM) had higher affinity than the (-)enantiomer (Ki = 13.9 nM) using synaptosomal membranes. The data obtained from [^{3}H]MK-801 binding to synaptosomal membranes were also similar to those for whole membranes ((+)enantiomer; K_D = 3.4 nM, (-)enantiomer; Ki = 15.3 nM) (Table 2.1). These results are consistent with previous observations that (+)MK-801 (K_D = 3 nM) has higher affinity than (-)MK-801 (Ki = 15 nM) using rat cortical membranes and that the [^{3}H]MK-801 binding site is therefore stereoselective (Wong et al., 1986).

For further characterisation of the interaction of (+)FR with the NMDA receptor, it was decided to synthesise tritium-labelled (+)FR. However (+)Cl-FR (Fig.2.1), the precursor compound for [^{3}H](+)FR, was only 4-6 times less potent than (+)FR with a Ki value of 548.4 ± 65.9 nM and 240.6 ± 78.0 nM in the absence and presence of added L-glutamate, respectively (Table 2.2, Fig.2.5). Therefore, it was necessary to have a method for purifying of [^{3}H](+)FR which would give good separation of (+)FR from (+)Cl-FR and also from potential autoradiolysis products which may be produced during radiosynthesis or storage of the [^{3}H]ligand.

2.3.3 Preparation and Purification of [^{3}H]FR115427

Prior to synthesis of [^{3}H](+)FR, an open column method for separating (+)FR and (+)Cl-FR using 200 µg of unlabelled compounds, (+)FR(free base) and (+)Cl-FR(free base), was evaluated. Since the intention was to purify batches of about

TABLE 2.2 : Effects of FR115427 Analogues on [^{3}H]MK-801 Binding to Rat Cortical Synaptosomal Membranes

	Ki (nM)	
	No addition	L-Glutamate
(+)FR (HCl salt) (FR115427)	114.5 ± 30.8	40.0 ± 2.1
(+)FR (TFA salt)	170.7 ± 44.1	50.2 ± 7.1
(+)FR (free base)	113.7 ± 54.3	37.5 ± 10.3
(+)Cl-FR (HCl salt)	548.4 ± 65.9	240.6 ± 78.0

The affinity of FR115427 analogues for [^{3}H]MK-801 binding sites on rat cortical synaptosomal membranes was studied in the absence or presence of added L-glutamate (10 μM) in the assay buffer. Membranes were incubated with [^{3}H]MK-801 (1 nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Ki values were calculated from the equation $Ki = IC_{50} / (1 + L/K_D)$. Values are mean ± S.E.M. of at least three independent experiments.

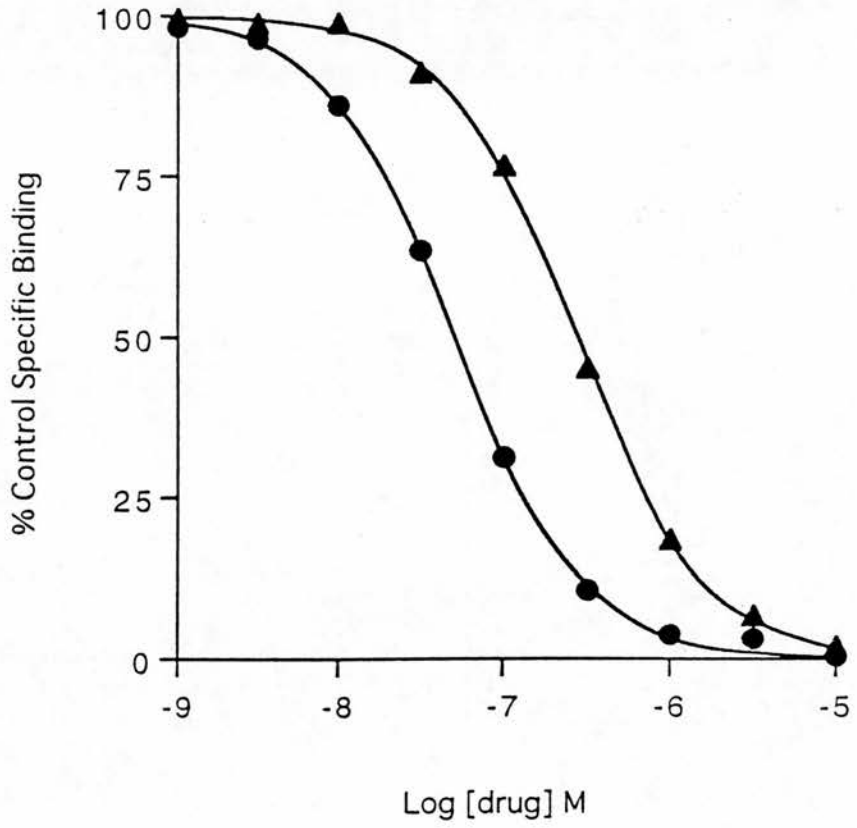


Fig. 2.5 Inhibition of [^{3}H]MK-801 binding to synaptosomal membranes by (+)Cl-FR and (+)FR. Cortical synaptosomal membranes were incubated with [^{3}H]MK-801 (1nM) in the absence or presence of increasing concentrations of test drug for 45 min at 25°C. Assays were carried out in the presence of added L-glutamate (10 μM). Non-specific binding was determined in the presence of 10 μM unlabelled (+)MK-801. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● (+)FR; ▲ (+)Cl-FR.

15 mCi of the crude radioactive material following tritiation, 200 μg of (+)FR and (+)Cl-FR therefore represent the amount of (+)FR expected to be present in 15 mCi of the crude radioactive material and the maximum amount of (+)Cl-FR depending on the success of the tritiation. After each fraction (350 μl) eluted from the column was freeze-dried, GDW (20 μl) was added to each fraction, then 5 μl was applied to TLC, and (+)FR and (+)Cl-FR were detected by U.V. light. As shown in fig.2.6, (+)FR started to elute at fraction 5 (1.4-1.75 ml) and the peak was at fractions 6 and 7 (1.75-2.45 ml) whereas (+)Cl-FR started to elute at fraction 9 (2.8-3.15 ml) with a peak at fractions 11 and 12 (3.5-4.2 ml). This eluting pattern for (+)FR and (+)Cl-FR was very similar in three separate experiments. Thus this open column method gave good separation of (+)FR from (+)Cl-FR and was also very reproducible, indicating that the method was suitable for purification of [^{3}H](+)FR.

Using the open column, 4 mCi of crude [^{3}H](+)FR synthesised from (+)Cl-FR was purified. Judging from fraction volumes (500 μl) used in this experiment and results with unlabelled (+)FR and (+)Cl-FR, it was expected that major peaks of [^{3}H](+)FR and (+)Cl-FR should have been in fractions 4 and 5, and fractions 7 and 8, respectively. As shown in fig.2.7, the radioactivity started to elute at fraction 5 (2-2.5 ml) and with major amount of [^{3}H]ligand in fractions 6-8 (2.5-4 ml, 95% of added radioactivity). Thus only one peak was observed with major fractions 6-8, indicating that almost no radiolysis products were produced during tritiation of (+)Cl-FR. Although major fractions (6-8) of [^{3}H]ligand were later than expected (fractions 4 and 5), it was shown by the open column and TLC

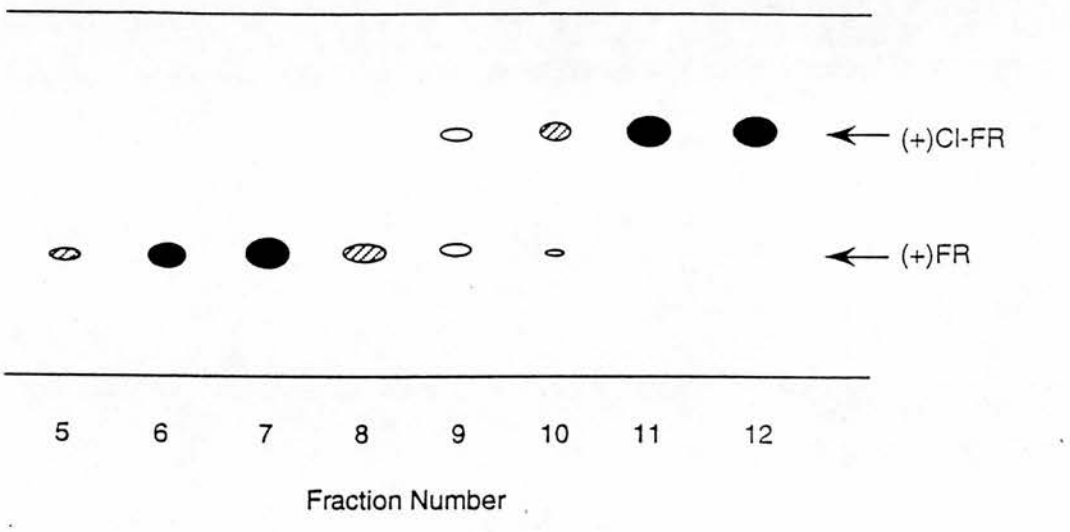


Fig. 2.6 TLC after separation of unlabelled (+)FR and (+)Cl-FR by open column method. Resin (Waters Preparative C-18, 55-105 μ,1.5 g) was applied onto disposal column (0.7 x 8 cm) and packed to 5 cm from the bottom of the column. Then unlabelled compound, (+)FR(free base) (200 μg/200 μl) and (+)Cl-FR (free base) (200 μg/200 μl) in solvent (CH_3CN : aq.0.1 % CF_3COOH (TFA) = 3 : 7) was applied to the column, and the eluting solution (350 μl/fraction) was collected. Each fraction was frozen on dry ice and the solvent evaporated by freeze drying. After addition of glass distilled water (20 μl) to each fraction, 5 μl was applied to silica gel thin layer chromatography (TLC) plates, developed ($CHCl_3$: MeOH = 9 : 1) and detected by U.V. light. Data are from a representive experiment.

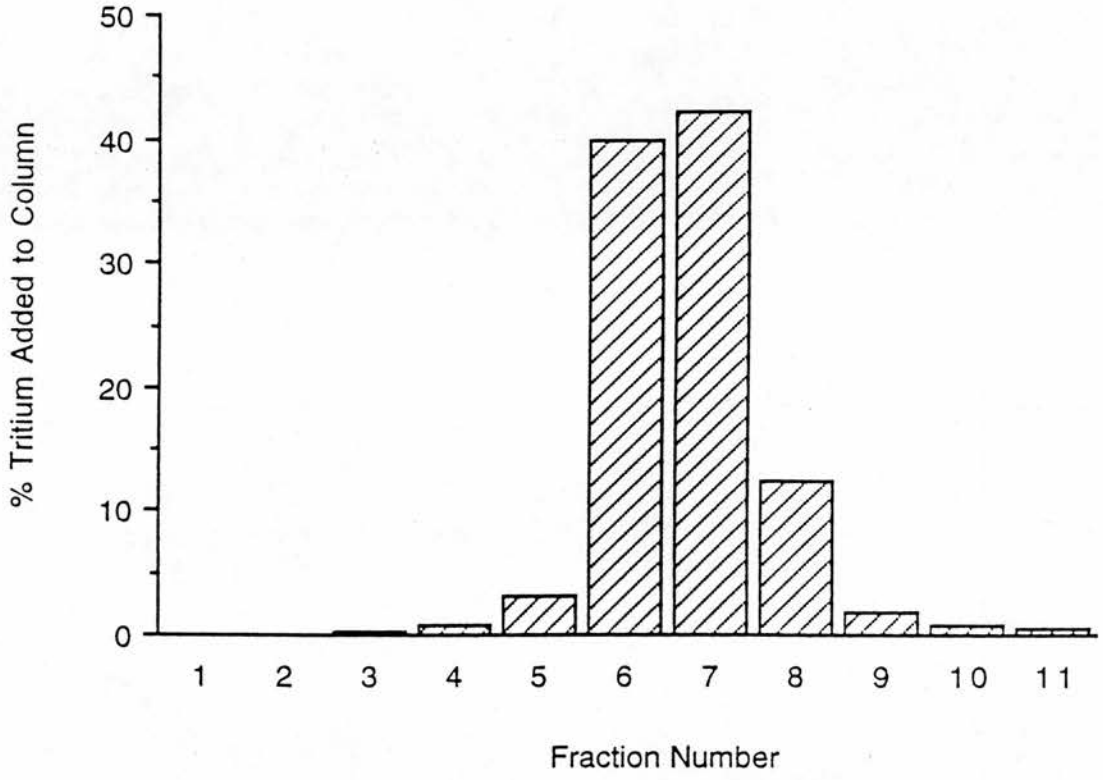


Fig. 2.7 Purification of [^{3}H](+)FR by open column. (+)Cl-FR (23.2 mg) was custom tritiated by Amersham International and stored at a radiochemical concentration of 10 mCi/ml in ethyl acetate. After removal of ethyl acetate, 4.02 mCi of crude [^{3}H](+)FR was dissolved in 402 μl with the eluting solvent (CH_3CN : aq.0.1 % CF_3COOH (TFA) = 3 : 7) then 4 mCi (400 μl) applied to the column. Fractions (500 μl) were collected and a subsample (2 μl) of each fraction was made up to 1 ml then 50 μl taken and diluted to 1 ml. Of this final dilution 100 μl was counted. Of the proportion, remaining (not added to the column) 2 μl was made up to 1 ml then 40 μl taken and diluted to 1 ml. Of this final dilution 100 μl was counted and these standards allowed the % of tritium recovered in each fraction to be calculated. The recovery from fractions 6 to 8 was calculated to be 95 %.

methods that fractions 6-8 did indeed correspond to (+)FR (see section 2.3.5). Fractions 6-8 were combined as [^{3}H](+)FR, and freeze-dried to remove the eluting solvent. After dilution to 10 μM in GDW, [^{3}H](+)FR was stored under liquid nitrogen until required.

After purification by open column, because CH_3CN-TFA is used as the eluting solvent of column [^{3}H](+)FR is obtained as a TFA salt. Therefore, the effects of different salts of (+)FR on [^{3}H]MK-801 binding to rat cortical synaptosomal membranes were examined.

2.3.4 Effects of Different (+)FR Salts on [^{3}H]MK-801 Binding to Rat Cortical Synaptosomal Membranes

Using synaptosomal membranes, the effects of the free base and the TFA salt of (+)FR on [^{3}H]MK-801 binding were examined in the absence and presence of added L-glutamate and compared with FR115427 (HCl salt of (+)FR) (Table 2.2). The HCl salt, TFA salt and free base of (+)FR inhibited [^{3}H]MK-801 binding to synaptosomal membranes with almost identical Ki values of 40.0 ± 2.1 nM, 50.2 ± 7.1 nM and 37.5 ± 10.3 nM in the presence of added L-glutamate, respectively. These Ki values were about 3-fold lower than the Ki values (HCl salt; 114.5 ± 30.8 nM, TFA salt; 170.7 ± 44.1 nM, Free base; 113.7 ± 54.3 nM) obtained in the absence of added L-glutamate. Statistical analysis by Dunnett's t-test showed that there was no significant difference in the affinity of (+)FR for [^{3}H]MK-801 binding in the absence and presence of added L-glutamate when measured using the free base, HCl or TFA salts.

2.3.5 Confirmation of [^{3}H](+)FR Purity

In the open column with unlabelled (+)FR (200 μg, approximately 900 nmol), (+)FR started to elute with 1.4 ml of solvent (Fig.2.6) whereas following application of crude [^{3}H](+)FR to the column (4 mCi, approximately 250 nmol) [^{3}H](+)FR started to elute with 2 ml of solvent (Fig.2.7). (+)Cl-FR (200 μg, approximately 900 nmol) started to elute with 2.8 ml solvent (Fig.2.6). The difference in the volume of solvent required to elute (+)FR may therefore depend on the amount of (+)FR applied to column. To clarify this, the open column separation was carried out with two different amounts (250 and 900 nmol) of unlabelled (+)FR each containing 4 μCi [^{3}H](+)FR and in parallel with a small amount using 4 μCi [^{3}H](+)FR only (0.25 nmol) (Fig.2.8). For the columns with 0.25 nmol and 250 nmol of (+)FR, (+)FR started to elute at 2.5 ml of solvent with the peaks at fractions 7 and 6, respectively. On the other hand, 2 ml of solvent was sufficient to start elution of (+)FR for the column with 900 nmol of (+)FR . These results suggest that the amount of (+)FR applied to the column affects to some extent the eluting pattern and that fractions 6-8 in the column with 4 mCi (250 nmol) of crude [^{3}H](+)FR (Fig.2.7) correspond to elution of authentic (+)FR.

The 4 mCi of [^{3}H](+)FR purified using the open column method was also checked using silica gel TLC (Fig.2.9). When 3 μl of [^{3}H](+)FR (1.78 μCi/ml) overloaded with 3 μl (10 mM) unlabelled (+)FR in glass distilled water were applied to the TLC plate, two peaks were observed. The major peak (69% of added [^{3}H](+)FR) was determined as (+)FR by U.V. detection at strip numbers 4 and 5, and the other, minor peak, corresponded to the

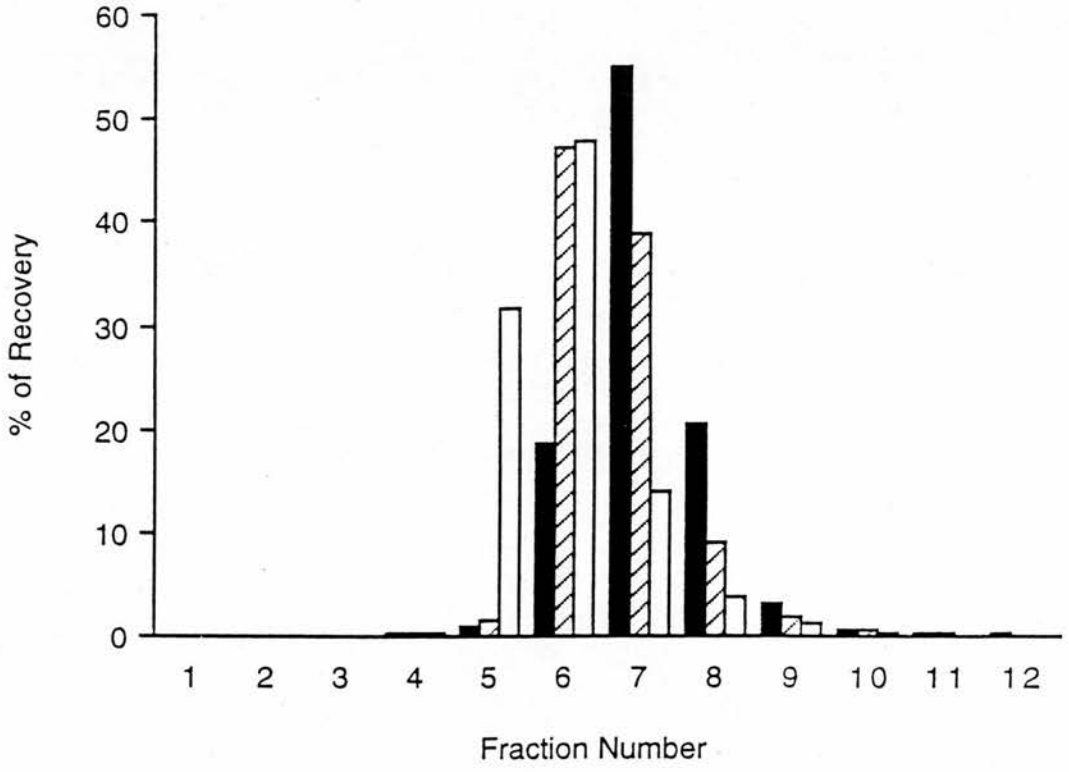


Fig. 2.8 Check of purity of [^{3}H](+)FR by open column. In order to get three different amounts (0.25, 250 and 900 nmol/400 μl respectively) of (+)FR, purified [^{3}H](+)FR (20 μCi/100 μl) was made up to 1 ml, then 200 μl (equivalent to 0.25 nmol) taken and diluted to 400 μl by the eluting solvent (CH_3CN : aq.0.1 % CF_3COOH (TFA) = 3 : 7) or the solvent containing an appropriate amount of unlabelled (+)FR (1 mg/ml). These different amounts was applied to each column then fractions (500 μl) were collected. A subsample (5 μl) of each fraction was taken for scintillation counting to determine recovery. ■ 0.25 nmol; ▨ 250 nmol; □ 900 nmol.

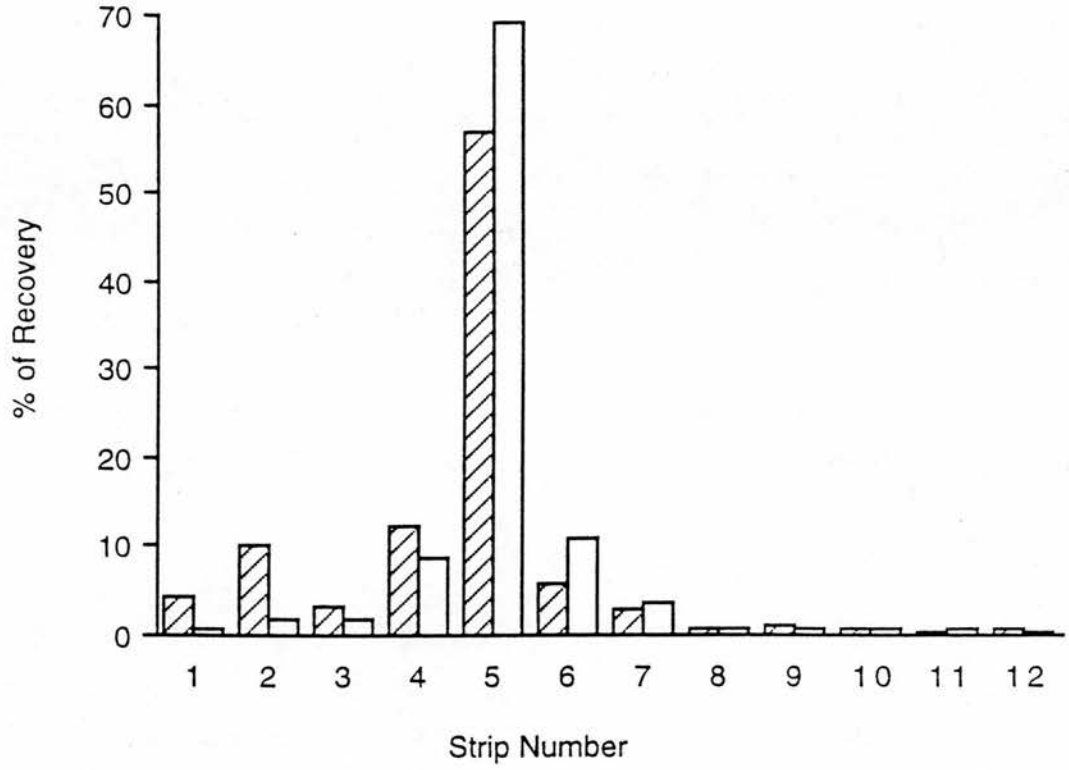


Fig. 2.9 Confirmation of [^{3}H](+)FR purity by TLC. Each 3 or 2 μl of (1.78 μCi/ml) [^{3}H](+)FR and 3 or 2 μl respectively 10 mM unlabelled (+)FR as carrier was spotted on TLC plates. Following development by $CHCl_3$-MeOH (9:1), the TLC plate was cut into 3 mm by 10 mm strips parallel to the origin. Each strip was transferred to a scintillation vial and 300 μl glass distilled water was added to elute the radioactivity. After 1 hr, 4 ml scintillation fluid added and the radioactivity in each strip was determined by liquid scintillation counting. The radioactivity in each strip was calculated as a recovery % from the total radioactivity applied to the origin by counting 3 or 2 μl of (1.78 μCi/ml) [^{3}H](+)FR as standards. Immediately before cutting of TLC, the spot of (+)FR was detected under U.V. light, its Rf value and the strip numbers corresponding to the spot noted. ▨ 3 μl; □ 2 μl.

origin. On the other hand, only one major peak (91% of added [^{3}H](+)FR) corresponding to (+)FR was observed when 2 µl of labelled and unlabelled (+)FR was applied. This result indicates that it is better to apply the compound in water in a small an amount as possible for checking purity by TLC. In addition, these results also suggest that a tiny amount of compound is not developed by the solvent (15% 3 µl samples; 3% 2 µl samples) due to damage of the surface of the silica gel at the origin by water. Nevertheless, the purity of [^{3}H](+)FR was shown to be greater than 90% by the TLC method using 2 µl each of [^{3}H](+)FR and unlabelled (+)FR (Fig.2.9). This compares well with the open column check of [^{3}H](+)FR purity where, for example, in the 250 nmol and 900 nmol column, 95% and 94% respectively corresponded to fractions containing authentic (+)FR.

2.3.6 In Vivo Distribution of [^{3}H](+)FR

The time course of the distribution of [^{3}H](+)FR after intravenous injection to rat was examined. Under anaesthesia, rats were injected with 500 µl of saline containing [^{3}H](+)FR (50 µCi, 3.2 nmol). This dose (approximately 1 µg) is much lower than the minimum dose (100 µg) required for behavioural effects, i.e. 1/100th. The blood level of radioactivity collected via the femoral artery or inferior vena cava is shown in table 2.3 and fig.2.10. The arterial blood levels of radioactivity were similar to those from the venous blood at 15, 30, 60, 90 and 180 min after [^{3}H](+)FR injection (Table 2.3). In blood, the peak level of radioactivity was obtained within 1 min and a rapid clearance of radioactivity was observed. After intravenous administration, the blood level was 0.148 %D/ml at 1 min,

TABLE 2.3 : In Vivo Distribution of Radioactivity in Blood after [^{3}H](+)FR Injection

	% injected dose / ml									
	time (min)									
	1	3	5	10	15	30	45	60	90	180
a)	0.148 (2)	0.087 (2)	0.066 (2)	0.052 (2)	0.067±0.01 (5)	0.048±0.004 (5)	0.044 (2)	0.045±0.001 (5)	0.053±0.004 (3)	0.052±0.002 (3)
b)	-	-	-	-	0.061±0.001 (3)	0.061±0.002 (3)	-	0.054 (2)	0.050±0.001 (3)	0.058±0.003 (3)

Rats were injected with 50 μCi [^{3}H](+)FR via the left femoral vein, and the blood was collected at various time points from a) femoral artery or b) inferior vena cava. Data are the mean value ± S.E.M. of 3-5 animals or the mean value of 2 animals. The number of animals is shown in parentheses.

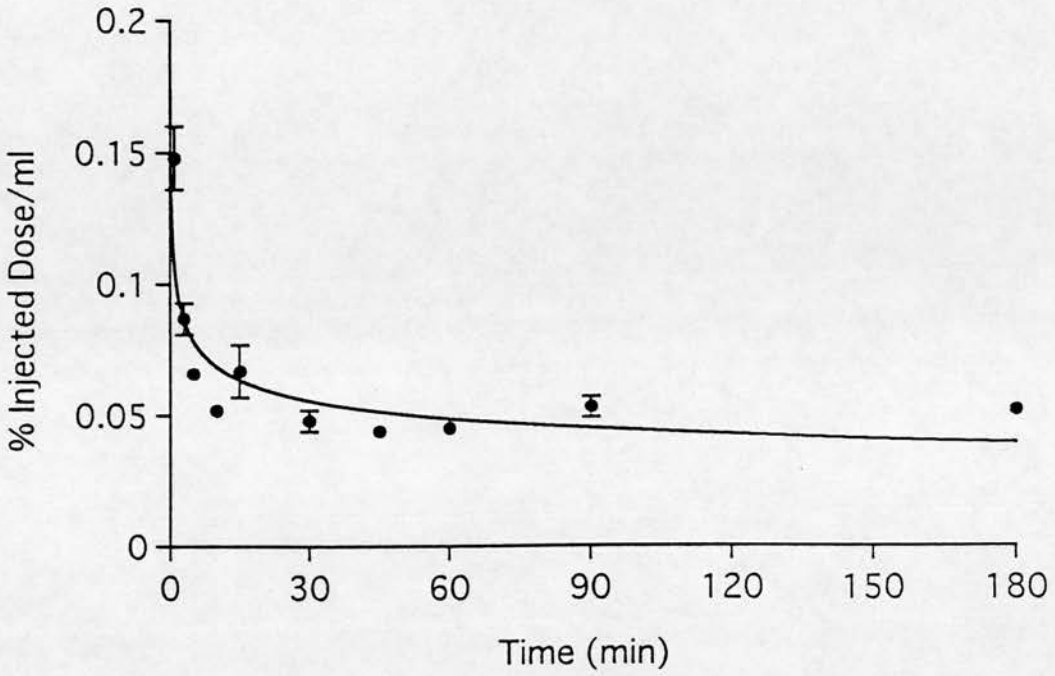


Fig. 2.10 Time course of radioactivity in arterial blood after [3H](+)FR injection. Under anaesthesia, rats were injected with 500 μl of saline containing 50 μCi (3.2 nmol) of [3H](+)FR through the cannula placed in the left femoral vein. Arterial blood (300 μl) was collected at various time points (1, 3, 5, 10, 15, 30, 45, 60, 90 and 180 min after injection) via femoral artery and a 200 μl sample was transferred to glass scintillation vials, and 1ml of Soluene-350 was added and left for 2hrs. 0.5 ml of 30% H_2O_2 was then added to bleach the blood and left for 1hr. After incubation of vials at 40°C for 30min, 10ml of Packard Hionic-Fluor was added and the radioactivity was measured by liquid scintillation spectrometry. Data are expressed as % injected dose per ml from 2-5 animals. In order to calculate the exact amount of [3H](+)FR injected to rats, standards were obtained by diluting of original radioactivity 1 : 200 and counting 100 μl in quadruplicate. Each time point is the mean value ± S.E.M. of 3-5 or range for 2 animals. The number of animals is shown in table 2.3.

followed by a biphasic decline with half lives of 6.513 min (α) and 2310 min (β) (Table 2.6). The AUC for blood was 9.537 %D · min/ml. The tissue levels of radioactivity at 15, 30, 60, 90 and 180 min after [^{3}H](+)FR injection are shown in tables 2.4, 2.5 and figs.2.11, 2.12, 2.13. In heart, kidney and liver, peak levels were obtained by 15 min (Table 2.4, Fig.2.11). At 15, 30 and 60 min after [^{3}H](+)FR injection, the 3 tissues contained radioactivity concentrations greater than that of blood. Liver had the highest concentrations of radioactivity when compared with kidney and heart as indicated by their AUC values (liver : 169.520, kidney : 87.920, heart : 27.042 %D · min/g) (Table 2.6). The clearance of radioactivity from liver was slower than from kidney and heart as indicated by their $t_{1/2}$ values (liver : 191.133 min, kidney : 75.016 min, heart : 83.104 min).

Uptake of [^{3}H](+)FR into whole brain reached a maximum by 15 min with 0.629 %D/g (Table 2.5, Fig.2.12). The radioactivity in whole brain (0.12 %D/g) was still observed even 3 hr after injection. At 15, 30 and 60 min after [^{3}H](+)FR injection, whole brain contained radioactivity concentrations greater than those of blood and heart, and lower than those of kidney and liver (Table 2.3, 2.4, 2.5, Fig.2.10, 2.11, 2.12). In all seven brain regions examined, the amount of radioactivity was highest at 15 min after injection (Table 2.5, Fig.2.13). All brain regions had similar radioactivity elimination half-lives with a value of approximately 50 min (Table 2.6). At all time points, although a significant difference could not be shown, the radioactivity concentration in the cerebellum was lower in comparison with other brain regions. The AUC value (46.001 %D · min/g) for

TABLE 2.4 : In Vivo Distribution of Radioactivity in Heart, Kidney and Liver after [^{3}H](+)FR Injection

	% injected dose / g tissue				
	time (min)				
Tissue	15	30	60	90	180
Heart	0.236 ± 0.003	0.192 ± 0.003	0.156	0.098 ± 0.001	0.080 ± 0.003
Kidney	0.823 ± 0.060	0.663 ± 0.040	0.490	0.319 ± 0.007	0.265 ± 0.019
Liver	1.008 ± 0.128	0.903 ± 0.074	0.650	0.672 ± 0.010	0.584 ± 0.037

Rats were injected with 50 μCi [^{3}H](+)FR via femoral vein and killed at various time points. Data are the mean value ± S.E.M. of 3 animals with the exception of the 60 min group (n = 2).

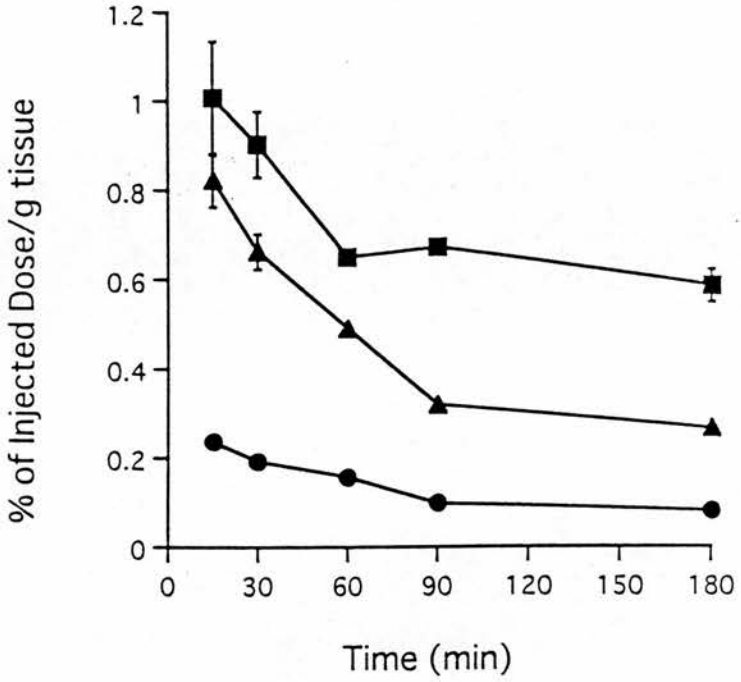


Fig. 2.11 Time course of radioactivity in heart, kidney and liver after [^{3}H](+)FR injection. Under anaesthesia, rats were injected with 500 μl of saline containing 50 μCi (3.2 nmol) of [^{3}H](+)FR through the cannula placed in the left femoral vein. At various times post-injection (15, 30, 60, 90 and 180 min), rats were killed, and liver, kidney and heart were removed, blotted dry and weighed. Samples 120 to 400 mg of each organ were taken, again blotted to removed as much blood or urine as possible and transferred to glass scintillation vials and dissolved with tissue solubilizer (Soluene-350, 1 ml/200 mg) at 37°C for 2 days. After being cooled to room temperature, 10 ml of Packard Hionic-Fluor scintillation fluid was added to each sample and radioactivity determined in a liquid scintillation analyser. Data are expressed as % injected dose per g tissue from 3 animals with the exception of the 60 min group (n=2). In order to calculate the exact amount of [^{3}H](+)FR injected to rats, standards were obtained by diluting of original radioactivity 1 : 200 and counting 100 μl in quadruplicate. Each time point is the mean value ± S.E.M. of 3 or range for 2 animals. ● Heart; ▲ Kidney; ■ Liver.

TABLE 2.5 : In Vivo Distribution of Radioactivity in Brain after [3H](+)FR Injection

a) % injected dose / g tissue

b) fmol / mg tissue

Brain region	time (min)				
	15	30	60	90	180
Whole brain	a) 0.629 ± 0.028	0.499 ± 0.023	0.339	0.178 ± 0.006	0.120 ± 0.003
	b) 18.992 ± 0.836	15.073 ± 0.686	10.226	5.383 ± 0.176	3.623 ± 0.083
Rostral cortex	a) 0.643 ± 0.033 (1.17)	0.526 ± 0.028 (1.20)	0.369 (1.24)	0.190 ± 0.008 (1.24)	0.123 ± 0.003 (1.10)
	b) 19.413 ± 0.992	15.887 ± 0.855	11.136	5.723 ± 0.241	3.702 ± 0.102
Caudal cortex	a) 0.621 ± 0.037 (1.13)	0.523 ± 0.018 (1.19)	0.362 (1.22)	0.190 ± 0.005 (1.24)	0.124 ± 0.002 (1.16)
	b) 18.743 ± 1.102	15.798 ± 0.553	10.937	5.731 ± 0.139	3.730 ± 0.049
Hippocampus	a) 0.614 ± 0.034 (1.12)	0.508 ± 0.023 (1.16)	0.367 (1.23)	0.205 ± 0.005 (1.34)	0.129 ± 0.005 (1.21)
	b) 18.539 ± 1.018	15.340 ± 0.697	11.083	6.189 ± 0.142	3.888 ± 0.136
Striatum	a) 0.645 ± 0.030 (1.18)	0.503 ± 0.019 (1.14)	0.359 (1.21)	0.183 ± 0.008 (1.20)	0.126 ± 0.004 (1.18)
	b) 19.489 ± 0.915	15.201 ± 0.572	10.837	5.531 ± 0.252	3.813 ± 0.111
Brainstem	a) 0.678 ± 0.025 (1.24)	0.476 ± 0.021 (1.08)	0.308 (1.03)	0.167 ± 0.008 (1.09)	0.117 ± 0.001 (1.09)
	b) 20.482 ± 0.761	14.383 ± 0.644	9.307	5.052 ± 0.233	3.546 ± 0.032
Midbrain	a) 0.676 ± 0.028 (1.23)	0.497 ± 0.023 (1.13)	0.317 (1.06)	0.169 ± 0.005 (1.11)	0.120 ± 0.004 (1.12)
	b) 20.413 ± 0.849	15.003 ± 0.684	9.584	5.093 ± 0.144	3.609 ± 0.122
Cerebellum	a) 0.548 ± 0.026	0.440 ± 0.020	0.298	0.153 ± 0.006	0.107 ± 0.004
	b) 16.539 ± 0.770	13.282 ± 0.613	9.006	4.614 ± 0.192	3.243 ± 0.128

Rats were injected with 50 μCi [3H](+)FR via femoral vein and killed at various time points. Data are expressed as a)% injected dose / g tissue and b) fmol / mg tissue, and are the mean value ± S.E.M. of 3 animals with the exception of the 60 min group (n = 2). The ratios (brain region / cerebellum) are shown in parentheses.

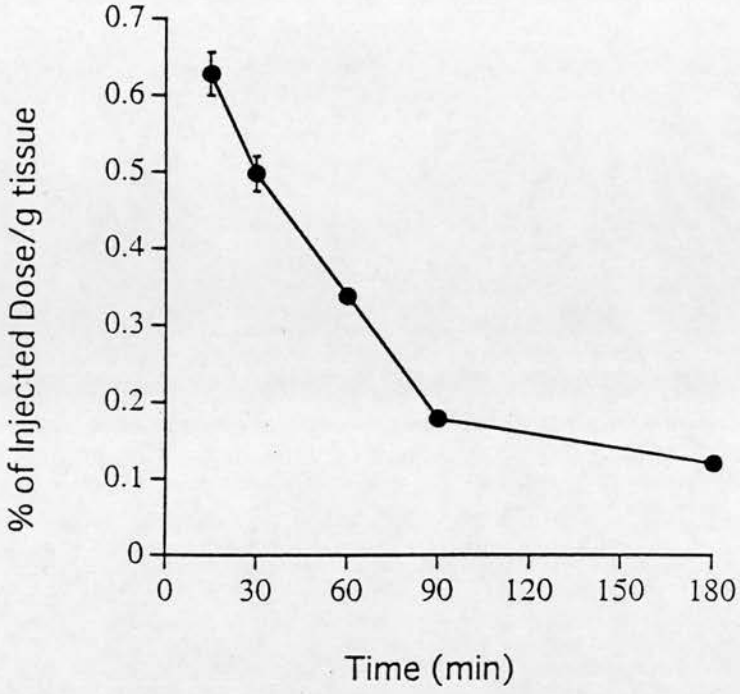


Fig. 2.12 Time course of radioactivity in whole brain after [^{3}H](+)FR injection. Under anaesthesia, rats were injected over 30 sec with 500 μl of saline containing 50 μCi (3.2 nmol) of [^{3}H](+)FR through the cannula placed in the left femoral vein. At various times post-injection (15, 30, 60, 90 and 180 min), the rats were killed by decapitation. The brains were removed, divided into 7 regions : rostral cortex, caudal cortex, hippocampus, striatum, brainstem, midbrain and cerebellum. The tissues were weighed in glass scintillation vials and dissolved with tissue solubilizer (Soluene-350, 1 ml/200 mg) at 37°C for 2 days. After being cooled to room temperature, 10 ml of Packard Hionic-Fluor scintillation fluid was added to each sample and radioactivity determined in a liquid scintillation spectrometry. The data for whole brain was obtained from total amounts of radioactivity and weight of all brain regions. In order to calculate the exact amount of [^{3}H](+)FR injected to rats, standards were obtained by diluting of original radioactivity 1 : 200 and counting 100 μl in quadruplicate. Data are expressed as % injected dose per g tissue from 3 animals with the exception of the 60 min group (n=2). Each time point is the mean value ± S.E.M. of 3 or range for 2 animals.

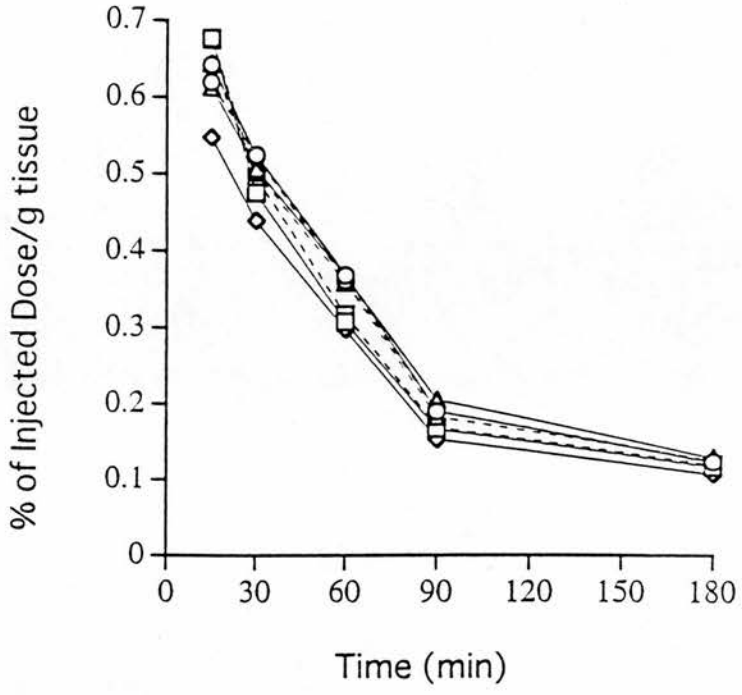


Fig. 2.13 Time course of radioactivity in brain regions after [^{3}H](+)FR injection. Under anaesthesia, rats were injected over 30 sec with 500 μl of saline containing 50 μCi (3.2 nmol) of [^{3}H](+)FR through the cannula placed in the left femoral vein. At various times post-injection (15, 30, 60, 90 and 180 min), the rats were killed by decapitation. The brains were removed, divided into 7 regions : rostral cortex, cauidal cortex, hippocampus, striatum, brainstem, midbrain and cerebellum. The tissues were weighed in glass scintillation vials and dissolved with tissue solubilizer (Soluene-350, 1 ml/200 mg) at 37°C for 2 days. After being cooled to room temperature, 10 ml of Packard Hionic-Fluor scintillation fluid was added to each sample and radioactivity determined in a liquid scintillation spectrometry. In order to calculate the exact amount of [^{3}H](+)FR injected to rats, standards were obtained by diluting of original radioactivity 1 : 200 and counting 100 μl in quadruplicate. . Data are expressed as % injected dose per g tissue from 3 animals with the exception of the 60 min group (n=2). Each time point is the mean value of 3 or 2 animals. —○— Rostral cortex; --○-- Caudal cortex; —△— Hippocampus; --△-- Striatum; —□— Brainstem; --□-- Midbrain; —◇—Cerebellum.

TABLE 2.6 : Pharmacokinetic Parameters of [^{3}H](+)FR in Rats After Injection

	Co (% D/g or ml)	Ke (min^{-1})	$t_{1/2}$ (min)	AUC (% D · min/g or ml)
Whole brain	0.765	0.01398	49.593	52.475
Rostral cortex	0.782	0.01334	51.958	52.475
Caudal cortex	0.755	0.01290	53.720	55.741
Hippocampus	0.729	0.01205	57.503	57.189
Striatum	0.777	0.01368	50.657	55.741
Midbrain	0.838	0.01614	42.939	55.741
Brainstem	0.838	0.01666	41.605	55.741
Cerebellum	0.668	0.01392	49.800	46.001
Heart	0.255	0.00834	83.104	27.042
Kidney	0.898	0.00924	75.016	87.920
Liver	0.992	0.00363	191.133	169.520
Blood	0.135	0.1064	6.513	9.537
	0.052	0.0003	2310	

The data of radioactivity in blood after [^{3}H](+)FR injection were fitted to biexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-\alpha t)} + B \times e^{(-\beta t)}$, where t is time after administration, Y is the measured radioactivity, A and B are the coefficients of the exponential terms, α is the apparent distribution rate constant, β is the apparent terminal rate constant. The data of radioactivity in brain and peripheral tissues were fitted to monoexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-Ke \cdot t)}$, where Ke is the elimination rate constant. Co : radioactivity at t = 0, $t_{1/2}$ (half-life) = 0.693/Ke or α or β, AUC : the area under the radioactivity concentration-time curve from zero to time infinity.

cerebellum was smallest among the seven brain regions (Table 2.6).

2.4 DISCUSSION

In the present experiments, the effects of (+)FR, (+)MK-801 and their enantiomers on [^{3}H]MK-801 binding to rat cortical whole membranes and synaptosomal membranes was examined in the absence and presence of added L-glutamate (10 μM) (Table 2.1). In order to examine the interaction of (+)FR with all NMDA receptors including non-neuronal and synaptic NMDA receptors, the two preparations (whole and synaptosomal membranes) were used for [^{3}H]MK-801 binding. In the presence of added L-glutamate, the K_D values in two membrane preparation (whole membranes; 3.4 ± 0.2 nM, synaptosomal membranes; 3.3 ± 0.6 nM) was almost the same with Bmax values of 74.3 ± 8.2 fmol/mg tissue (whole membranes) and 48.8 ± 5.9 fmol/mg tissue (synaptosomal membranes). The Bmax values for the two tissue preparations cannot be directly compared since they are not expressed as per mg of protein, and protein recovery in the synaptosomal membrane preparation is normally only about 50% of that of the whole membrane preparation. Indeed a higher density of NMDA receptors on a mg of protein basis would be anticipated for the synaptosomal membranes. In the presence of added L-glutamate, the Ki values of (+)FR in two membrane preparations (whole membranes; 48.1 ± 5.9 nM, synaptosomal membranes; 40.0 ± 2.1 nM) was almost the same. Addition of L-glutamate produced an increase in affinity of (+)FR and (+)MK-801 for [^{3}H]MK-801 binding sites in both brain membrane preparations. Binding of L-glutamate to the NMDA

receptor presumably causes a conformational change that facilitates the interaction of (+)MK-801 with its binding site within the NMDA receptor associated ion channel (Foster and Wong, 1987). This result suggests that (+)FR preferentially binds to an activated state of the NMDA receptor-ion channel complex and, like (+)MK-801, its binding site resides on or within the ion channel. The inhibitory effect of (+)FR on [^{3}H]MK-801 binding has been reported by Nakanishi et al. (1995) who also showed that (+)FR inhibited binding of [^{3}H]TCP but not [^{3}H]CPP or [^{3}H]glycine to rat brain membranes. In addition, systemic administration of (+)FR blocked NMDA-induced convulsions in mice, but not quisqualate- or kainate-induced convulsions (Nakanishi et al., 1995). These results are consistent with (+)FR, like MK-801, being a non-competitive NMDA antagonist.

The effect of L-glutamate was more apparent for synaptosomal membranes than for whole membranes. These results indicate that residual endogenous L-glutamate in the synaptosomal membranes was lower than that in the whole membranes. Foster and Wong (1987) showed the removal of endogenous glutamate and aspartate from rat cortical synaptosomal membranes by repeated washing reduces the affinity of [^{3}H]MK-801 for its binding site. According to their report, the K_D value for [^{3}H]MK-801 binding to synaptosomal membranes before washing was 2.2 nM whereas the K_D value for the membranes after washing was 16.5 nM. In addition, they demonstrated that glutamate and aspartate levels in synaptosomal membranes before washing were 2.98 and 3.67 nmol/mg protein respectively, whereas levels after washing

were 0.42 and 0.54 nmol/mg protein respectively. In the present experiments, the K_D value for [^{3}H]MK-801 binding to synaptosomal membranes was 3.3 nM and 25.0 nM in the presence and absence of added L-glutamate, respectively. These K_D values are consistent with the K_D values for synaptosomal membranes before and after washing shown by Foster and Wong (1987). Thus the extensively washed synaptosomal membranes used by Foster and Wong seem to correspond to the synaptosomal membranes used in the present experiment and to give similar K_D values, although there are some minor differences in the details of the membrane preparation protocols. They obtained crude synaptosomal membranes by three washes (suspension in distilled water and centrifugation; 50,000g, 20 min) of the P_2 pellet followed by resuspension in 5 mM Tris-HCl buffer and centrifugation. For well washed membranes, a further 4 cycles of washing with Tris-HCl buffer was carried out. In the present experiments, synaptosomal membranes were prepared by washing three times (suspension in distilled water and centrifugation; 50,000g, 10 min) and incubation at 37°C for 30 min (see section 2.2.1). Therefore inclusion of an incubation step appears to be a very efficient way of removing endogenous excitatory amino acids in comparison with repeated washings. Thus [^{3}H]MK-801 binding using well washed synaptosomal membranes has an advantage for facilitating study of L-glutamate modulation of NMDA receptors in comparison with binding using whole brain membranes.

The L-glutamate-induced increase in affinity of (+)FR for [^{3}H]MK-801 binding sites was smaller than that of (+)MK-801 in synaptosomal membranes although the precise significance of

this finding remains unclear. The affinity of (+)FR was increased by about 3-fold from 114.5 to 40.0 nM in the presence of added L-glutamate without significant difference ($p=0.1372$), whereas the affinity of (+)MK-801 was significantly ($p<0.05$) increased by about 8-fold from 25.0 to 3.3 nM (Table 2.1). This finding appears to be supported by results from [^{3}H](+)FR binding to rat cortical membranes (Sherriffs et al., 1993). It has been demonstrated that L-glutamate (10 μM) increases specific [^{3}H](+)FR and [^{3}H]MK-801 binding maximally to 438 and 592% of control binding, respectively. This difference suggests that (+)FR and (+)MK-801, as might be expected because they are different chemical structures, may not interact in the same manner with identical amino acid residues in the NMDA receptor ion channel. A common set of amino acid interactions in the binding site would however be anticipated. Recently, Hodgikiss et al. (1993) reported that the (+)FR and (+)MK-801 interact with a common binding site in electrophysiological experiment. In the presence of increasing concentrations of (+)FR, the density of [^{3}H]MK-801 binding sites was not altered whereas binding site affinity was reduced. Dixon analysis of these data confirmed that (+)FR competitively inhibited [^{3}H]MK-801 binding. Therefore the access of (+)FR to the same binding site may be less dependent on the ongoing activity of the NMDA receptor.

(+)FR inhibited the binding of [^{3}H]MK-801 although (+)FR was 16- and 12-fold less potent than (+)MK-801 in the presence of added L-glutamate (10 μM) using whole brain membranes and synaptosomal membranes, respectively. This result is consistent with data obtained from the NMDA-induced convulsion test in mice. (+)FR (0.32 μg, i.c.v.) completely inhibits

convulsion whereas only 0.032 μg (+)MK-801 is required, indicating that (+)FR is 10 times less potent than (+)MK-801 in this test (Nakanishi et al., 1995). Wong et al. (1988) showed the inhibition of [^{3}H]MK-801 binding to well washed synaptosomal membranes by known non-competitive NMDA antagonists. The order of potency for their antagonists was (+)MK-801 (K_D = 3 nM) > TCP (Ki = 14 nM) > PCP (Ki = 42 nM) >> ketamine (Ki = 1090 nM). In the present study with synaptosomal membranes in the presence of added L-glutamate, the K_D value for (+)MK-801 was 3.3 nM and the Ki value for (+)FR was 40 nM. Therefore it seems that (+)FR and PCP have a similar potency as inhibitors of [^{3}H]MK-801 binding to rat cortical membranes. Sherriffs et al. (1993) showed that a number of NMDA receptor channel blockers had very similar Ki values as inhibitors of both [^{3}H]MK-801 and [^{3}H](+)FR binding indicating that both ligands label the same site (Table 2.7). In addition, it was shown that the potency of (+)FR was similar to that of PCP (Table 2.7). This result appears to correspond to previous observations that (+)FR and PCP have anti-ischaemic effects at similar doses in the gerbil transient ischaemic model (Katsuta et al., unpublished data). Interestingly, the dose of (+)FR for producing PCP-like behaviour (e.g. head-weaving, turning, ataxia) in mice is significantly higher in comparison with PCP (Shirakawa et al., unpublished data), indicating that PCP-like behaviour induced by (+)FR and PCP may not only be due to antagonism of the NMDA receptor linked ion channels.

When compared with the corresponding (+)enantiomers, (-)FR and (-)MK-801 were respectively 98- and 4.2-fold less potent as inhibitors of [^{3}H]MK-801 binding to synaptosomal

TABLE 2.7 : Inhibition of [^{3}H](+)FR and [^{3}H]MK-801 Binding to Rat Cortical Synaptosomal Membranes by Non-competitive NMDA Receptor Antagonists

	K_D / Ki (nM)	
	[^{3}H](+)FR	[^{3}H]MK-801
(+)FR	45.4 ± 3.9	35.4 ± 3.8
(-)FR	4732 ± 395	3756± 227
(+)MK-801	6.45 ± 0.71	3.57 ± 0.4
(-)MK-801	18.4 ± 3.5	16.0 ± 1.6
PCP	35.8 ± 2.8	36.2 ± 4.5
Ketamine	576 ± 107	354 ± 59.0
N-Allylnormetazocine	304 ± 41.8	316 ± 24.8

Cortical synaptosomal membranes were incubated with 10 nM [^{3}H](+)FR (45 min) or 1 nM [^{3}H]MK-801 (120 min) in the presence of 10 μM L-glutamate and increasing concentrations of test drugs (0.1 nM - 300 μM). Non-specific binding was determined in the presence of 30 μM unlabelled ligand. Ki values were calculated from the equation $Ki = IC_{50} / (1 + L/K_D)$. Values are mean ± S.E.M. of at least three experiments performed in duplicate. (taken from Sherriffs et al., 1993)

membranes in the presence of added L-glutamate. Thus the two stereoisomers of FR showed a much larger difference in affinity when compared with the stereoisomers of MK-801. This phenomenon was also observed in the mouse NMDA-induced convulsion test (Nakanishi et al., 1995). For an inhibitory effect of i.c.v. injection of drugs on NMDA-induced convulsions, (-)FR is about 100 times less potent than (+)FR whereas (+)MK-801 and (-)MK-801 have almost the same potency. This difference is thought to be a reflection of the differences in their chemical structures and in particular to the quazi-symmetrical nature of the MK-801 molecule. At the chiral centre of FR, all four substituents are substantially different, whereas two of the groups at the chiral centre of MK-801 are rather similar (Fig.2.1).

In advance of the synthesis of [^{3}H](+)FR, the chlorinated aromatic precursor (+)Cl-FR was tested against [^{3}H]MK-801 binding to rat cortical synaptosomal membranes. (+)Cl-FR had a reasonably high affinity for [^{3}H]MK-801 binding sites, being about 5-fold less potent than (+)FR. Therefore for the small amount of (+)Cl-FR which potentially may remain after tritiation, a suitable method was required for its complete separation from (+)FR. As shown in fig.2.2, an open column method used in the present experiment gave good separation of (+)FR from (+)Cl-FR. This method is also expected to separate side products after synthesis of [^{3}H](+)FR and radiolysis products following storage of the material. Using this open column method, the crude [^{3}H](+)FR synthesised from (+)Cl-FR was purified and [^{3}H](+)FR was obtained as the TFA salt. (+)FR(TFA salt) had similar characteristics as an inhibitor of

[^{3}H]MK-801 binding when compared with FR115427 ((+)FR(HCl salt)), suggesting that [^{3}H](+)FR(TFA salt) is a suitable ligand for the binding studies. [^{3}H](+)FR obtained by an open column method had high purity (> 90%) as confirmed by TLC method. Thus the open column and TLC methods were useful for checking purity or decomposition of [^{3}H](+)FR during storage under liquid nitrogen.

Sherriffs et al. (1993) demonstrated that [^{3}H](+)FR can be used for the pharmacological investigation of the NMDA receptor ion channel. The [^{3}H](+)FR binding site density is similar to the Bmax value for [^{3}H]MK-801 in rat cortical synaptosomal membrane preparation. The K_D (45 nM) for [^{3}H](+)FR binding is in agreement with the Ki value (35 nM) for (+)FR measured in the [^{3}H]MK-801 binding assay. The affinities of other NMDA receptor channel blockers such as PCP and ketamine for the [^{3}H](+)FR binding site are consistent with binding to a similar site to that occupied by (+)MK-801. These results indicate that [^{3}H](+)FR and [^{3}H]MK-801 bind to a similar site within the NMDA receptor ion channel. Using [^{3}H](+)FR as the radioligand, (+)FR is approximately 100-fold more potent than (-)FR, whilst there is only a 3-fold separation in the affinities of the stereoisomers of MK-801, suggesting that the stereoselectivity of FR is much greater than that of MK-801. Thus radioligand binding studies using [^{3}H](+)FR are in agreement with the results in the present work.

Using purified [^{3}H](+)FR, its <u>in vivo</u> distribution after intravenous injection to rats was examined. Intravenous administration of [^{3}H](+)FR resulted in a rapid accumulation of radioactivity in rat brain, indicating that (+)FR can penetrate the

blood-brain barrier. The clearance of radioactivity from liver was slower than kidney and heart. At all time points used, although a significant difference could not be shown, the radioactivity concentrations in the cerebellum was lower in comparison with other brain regions. The ratios of the six brain regions to cerebellum were shown in table 2.5. Rostral cortex, caudal cortex, hippocampus and striatum had higher ratio than midbrain and brainstem with exception of the 15 min time point. In vitro autoradiography with [^{3}H]MK-801 in rat brain showed that the highest concentrations of [^{3}H]MK-801 binding sites occur in brain regions such as hippocampus and cerebral cortex, and very low levels are detected in brainstem and cerebellum (Bowery et al., 1988). In addition, an in vivo [^{3}H]MK-801 autoradiography study in mouse brain demonstrated regional selectivity with the highest density in hippocampus and cortex and with virtually no specific binding in the cerebellum (Price et al., 1988b). However in the present study it is not clear if the [^{3}H](+)FR represented binding to NMDA receptors. For this it would be necessary to see if the relative distribution of [^{3}H](+)FR in cortex and hippocampus compared with cerebellum is altered as a result of saturation of the NMDA receptor ion channels with unlabelled drug. The time course of radioactivity in brain regions after [^{3}H](+)FR injection was similar when compared the time course of in vivo distribution of (+)-3-[^{125}I]iodo-MK-801 in rat brain regions (Gibson et al., 1992). Following intravenous administration of (+)-3-[^{125}I]iodo-MK-801, localisation in 6 brain regions is rapid with peak concentrations occur within 30 min, and the distributions of radioactivity at 30, 60, 120 and 240 min post-injection are

0.6-1.1, 0.3-0.5, 0.1-0.2 and 0.1-0.2 %D/g, respectively. The blockade of localisation of (+)-3-[^{125}I]iodo-MK-801 by unlabelled MK-801 is demonstrable when examined 4 hr post-injection, sufficient time to permit washout of the non-specifically bound radioligand, indicating that (+)-3-[^{125}I]iodo-MK-801 may be a useful in vivo imaging agent for the NMDA receptor-channel complex (Gibson et al., 1992). However Gibson et al. (1992) concluded that (+)-3-[^{125}I]iodo-MK-801 is not ideal to provide a means of quantitative evaluation of the NMDA receptor-channel complex because of very high non-specific binding. Blin et al. (1991) reported PET studies using ^{18}F-labelled fluoro-methyl-MK-801 ([^{18}F]FMM) in baboons. A differential retention of [^{18}F]FMM exists in the cerebral cortex and striatum relative to cerebellum, with region/cerebellum ratios values ranging from 1.1 to 1.6. These ratios values are compatible with those for [^{3}H](+)FR (rostral cortex/cerebellum = 1.15-1.24, striatum/cerebellum = 1.14-1.21). Pretreatment with pharmacological doses of MK-801 or PCP was not able to clearly alter [^{18}F]FMM brain regional kinetics, indicating an extremely high non-specific binding (Blin et al., 1991).

In conclusion, [^{3}H](+)FR may be a useful tool for in vivo imaging of NMDA receptors. However it will be important to determine if the higher concentrations (which are quite small) in brain regions such as rostral/caudal cortex and hippocampus compared with cerebellum are due to in vivo specific binding of [^{3}H](+)FR to the NMDA receptor or for example to differences in pharmacokinetics of [^{3}H](+)FR in cerebellum. It may be possible to do this by pretreatment with unlabelled drug ((+)FR or (+)MK-801) at a dose sufficient to saturate the receptors prior

to injection with [^{3}H](+)FR, as was carried out for [^{125}I]5-IC (see chapter 3 section 3.2.9). In addition, it will be important to confirm that the radioactivity in the brain is authentic [^{3}H](+)FR using for example the open column method or TLC. As described in chapter 1, it is well established that the in vitro binding of MK-801 is markedly enhanced in the presence of glutamate, and extracellular levels of glutamate are increased in cerebral ischaemia. It has been suggested that in vivo autoradiographic studies with (+)-3-[^{125}I]iodo-MK-801 show increased receptor-mediated binding in ischaemic rat brain (McCulloch and Iversen, 1991). In ischaemic tissues, specific binding of (+)-3-[^{125}I]iodo-MK-801 is 4-fold that of non-specific binding to the cerebellum. Thus (+)-3-[^{125}I]iodo-MK-801 might be useful as a possible SPET ligand under ischaemic conditions such as stroke. Therefore it would be interesting to examine the in vivo distribution of [^{3}H](+)FR under cerebral ischaemic conditions in anticipation of the possible future development of a close structural analogue as a SPET ligand.

CHAPTER 3

DEVELOPMENT OF PHOTOAFFINITY AND SPET LIGANDS FOR THE 5-HT TRANSPORTER

3.1 INTRODUCTION

The radiolabelled forms of selective 5-HT uptake inhibitors have been used in ligand binding experiments for characterisation of the 5-HT transporter in blood platelets and brain. [^{3}H]Imipramine was the first blocker to be developed as a 5-HT transporter radioligand, but it was reported to bind to high (K_D = 6.9 nM) and low (K_D = 292 nM) affinity sites in brain tissue (Hrdina, 1984). Tricyclic antidepressants effectively inhibit the high affinity component of [^{3}H]imipramine binding to rat cortical membranes with a rank order of potency which corresponds with their ability to block 5-HT uptake in vitro. In contrast, the order of potency of drugs tested as inhibitors of low affinity [^{3}H]imipramine binding, does not correlate with their ability to inhibit 5-HT uptake. Thus only the high affinity binding sites for [^{3}H]imipramine correspond to the 5-HT transporter. Recently more selective 5-HT uptake inhibitors such as [^{3}H]citalopram (D'Amato et al., 1987a), [^{3}H]paroxetine (Habert et al., 1985), [^{3}H]norzimelidine (Hall et al., 1982) and [^{3}H]indalpine (Bénavidès et al., 1985) were utilised to label 5-HT uptake sites in brain. In contrast to the [^{3}H]imipramine binding assay which must be conducted at 0°C, presumably to slow dissociation of the ligand from the transporter, [^{3}H]paroxetine and [^{3}H]citalopram binding can be conducted at higher temperatures (25°C or 37°C) (Habert et al., 1985; D'Amato et al., 1987a). This property could be important for characterisation of the 5-HT transporter and drug interactions under physiological conditions. It has been shown that both [^{3}H]citalopram and [^{3}H]paroxetine bind selectively with very high affinity (low nanomolar range) to a single class of sites and that

the potencies of a range of drugs for inhibition of ligand binding and 5-HT uptake is closely correlated (D'Amato et al., 1987a; Habert et al., 1985; Marcusson et al., 1988) thus indicating that [^{3}H]paroxetine and [^{3}H]citalopram indeed selectively bind to the 5-HT transporter. In addition, ligand binding of both radioligands are almost completely abolished after lesioning of 5-HT neurons in rat brain whereas [^{3}H]imipramine binding is only decreased partially, indicating that the binding sites labelled by [^{3}H]paroxetine and [^{3}H]citalopram corresponds to the neuronal 5-HT transporter (D'Amato et al., 1987a; Habert et al., 1985; Marcusson et al., 1988). Thus [^{3}H]citalopram and [^{3}H]paroxetine are superior radioligands for the 5-HT transporter in comparison with [^{3}H]imipramine. Autoradiographic studies showed that the distribution pattern of [^{3}H]paroxetine and [^{3}H]citalopram binding in rat brain are consistent with the organisation of serotonergic terminals and cell bodies (DeSouza and Kuyatt, 1987; Hrdina et al., 1989; D'Amato et al., 1987a). In these studies, high binding densities were found in the brain stem nuclei, thalamus and hypothalamus, moderate binding in basal ganglia, cortex and hippocampus, whereas low binding density was detected in cerebellum. Dissociation of receptor-ligand complex can be studied either by extensive ("infinite") dilution of the sample or by addition of high amounts of unlabelled ligands (both of which really prevent further [^{3}H]ligand-receptor association), and these two procedures should theoretically give the same result. However it has been reported that the dissociation of [^{3}H]imipramine, [^{3}H]paroxetine and [^{3}H]citalopram from the 5-HT transporter are influenced by several drugs (Plenge and Mellerup, 1985; Plenge et al., 1990; Plenge et al., 1991). For example,

5-HT (200 μM) markedly decreased the dissociation rate of [^{3}H]imipramine, had a moderate effect on [^{3}H]paroxetine but very little effect on [^{3}H]citalopram dissociation from platelet membranes. Almost no dissociation of [^{3}H]paroxetine by 200 μM of citalopram and paroxetine is observed using rat brain membranes. In addition, the dissociation rate of [^{3}H]citalopram is decreased to a greater extent by citalopram than by paroxetine. These results indicate that the three ligands may not bind to identical domains on the 5-HT transporter. In human brain, the relative potencies of various compounds in inhibiting [^{3}H]paroxetine and [^{3}H]citalopram binding are identical to those in rat brain, suggesting that binding of both ligands in human brain exhibit the same pharmacological characteristics as in rat brain (Bäckström et al., 1989; D'Amato et al., 1987b). However, 80% of [^{3}H]citalopram binding in human cortex is 5-HT sensitive, whereas only 40% of [^{3}H]paroxetine binding is 5-HT sensitive, suggesting that the amount of specific binding of [^{3}H]citalopram is higher than that of [^{3}H]paroxetine (Bäckström et al., 1989; D'Amato et al., 1987b). Thus [^{3}H]citalopram may be a better radioligand for studying the 5-HT transporter in human brain.

Neuroimaging techniques such as SPET are potentially very useful methods for studying neurotransmitter receptors in the living human brain. The development of SPET ligands for the 5-HT transporter will establish powerful methods for diagnosis and monitoring the course of psychiatric disorders such as depression. However, no human studies for the 5-HT transporter have been reported so far. Previously, Wolfe et al. (1987) demonstrated that [^{3}H]cyanoimipramine can be given to rats to provide a measure of 5-HT uptake sites in the brain in vivo.

Recent work has demonstrated that [^{3}H]paroxetine and [^{3}H]citalopram are selective agents for in vivo labelling of cerebral 5-HT uptake sites (Scheffel and Hartig, 1989; Scheffel and Ricaurte, 1990; Hume et al., 1991). The in vivo distribution of binding of both radioligands in mouse and rat brain is consistent with the expected in vitro distribution, and the binding correlates closely to the localisation of 5-HT uptake sites. In addition, a profound reduction in in vivo [^{3}H]paroxetine binding was shown in brains of rats treated with the psychoactive recreation drug 3,4-methylenedioxy-methamphetamine (MDMA) (Sheffel and Ricaurte, 1990). This effect of MDMA was similar to results observed using in vitro [^{3}H]paroxetine binding in rat brain (Battaglia et al., 1987; Sharkey et al., 1991). These reports suggest that paroxetine and citalopram labelled with suitable radionuclides might be useful for studying the 5-HT transporter in living human brain using neuroimaging techniques such as PET and SPET.

The topography of the 5-HT transporter including a number of its amino acid groups was investigated by Langer and Graham (1989). Covalent modification of cystein residues by the sulphydryl-alkylating reagent N-ethylmaleiimide (NEM), or of tyrosine residues by the alkylating reagent p-nitrobenzyl-sulphonyl fluoride, produce large reductions in [^{3}H]paroxetine binding to rat cortical membranes. These results suggest that sulphydryl and tyrosine hydroxyl groups are located at, or very close to the paroxetine binding site on the 5-HT transporter. Treatment of the cortical membranes with trypsin and chymotrypsin also produces a reduction in [^{3}H]paroxetine binding, and [^{3}H]paroxetine binding is decreased after treatment

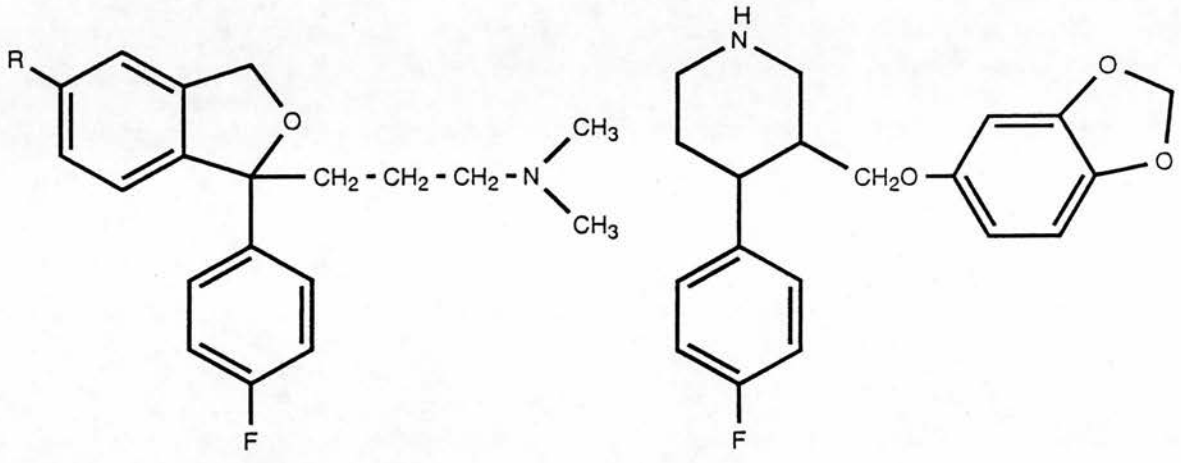
of membranes with phospholipase C and A_2. Thus the 5-HT transporter requires an intact protein and lipid environment in order to maintain its ability to bind [^{3}H]paroxetine. For characterisation and purification of the 5-HT transporter, it has been solubilised from rat cortical membranes using digitonin (Habert et al., 1986). The pharmacological profile of the solubilised transporter was similar to that of the intact transporter in cortical membranes. Analysis of the purified preparation showed a major polypeptide with a molecular weight of 110,000 (Langer and Graham,1989). Recently, the cloning of 5-HT transporter was reported by two groups (Blakely et al., 1991; Hoffman et al., 1991). The complementary DNA (cDNA) sequence from rat brain predicts a protein of 607 amino acids with a relative molecular mass of 68,000 and displays 11 or 12 transmembrane domains (Blakely et al., 1991). On the other hand, the study with a cDNA from rat basophilic leukemia cells predicts a protein of 653 amino acids with 12 to 13 putative transmembrane domains (Hoffman et al., 1991). The 5-HT transporter has significant homology to the GABA, dopamine and noradrenaline transporters. Both studies showed that the 5-HT transporter expressed from the cDNA has pharmacologically very similar characteristics to the rat brain 5-HT transporter, with high affinity sites for selective 5-HT uptake inhibitors such as citalopram and paroxetine.

Photoaffinity labelling is a powerful technique for biochemical investigation of receptors. Previously, Rotman and Pribluda (1982) demonstrated that [^{3}H]azido-imipramine, a photoaffinity ligand for the 5-HT transporter, binds covalently to guinea pig brain synaptosome preparations and labels two

main proteins with molecular weights of 60,000-65,000. Studies with more selective photoaffinity ligands than imipramine will provide useful information for understanding of the native 5-HT transporter.

In the present study, citalopram (Fig.3.1) rather than paroxetine was chosen as a prototype compound for both photoaffinity and SPET ligands. [^{3}H]Citalopram binds to a single site with a K_D value for the 5-HT transporter of about 1 nM although this is about 10-fold lower affinity than that of [^{3}H]paroxetine (D'Amato et al, 1987a; Marcusson et al, 1988). However citalopram has greater selectivity than paroxetine which has some affinity also for the noradrenaline transporter (Hyttel, 1982). In addition, of major importance was the ease of incorporation of site directed "atoms" into citalopram structure compared with paroxetine.

Bigler et al. (1977) reported the structure-activity relationships of citalopram analogues using <u>in vitro</u> [^{14}C]5-HT uptake into rabbit platelets. As shown in table 3.1, a halogen atom (F, Cl and Br) or a cyano group can be substituted on the 5 and 4' positions of citalopram without loss of biological activity. Therefore, citalopram analogues containing either an azide or acylazide moiety at the 5 or 4' position may be suitable photoaffinity ligands. Similarly, 5 or 4'-iodo-citalopram analogue might be expected to provide a SPET ligand. Therefore, 5-azido-citalopram (5-AC) (Fig.3.1) and 5-iodo-citalopram (5-IC) (Fig.3.1) were synthesised by Dr.I.M.Dawson in the Department of Pharmacology, University of Edinburgh as potential candidates for photoaffinity and SPET ligands, respectively.



Citalopram : R = CN

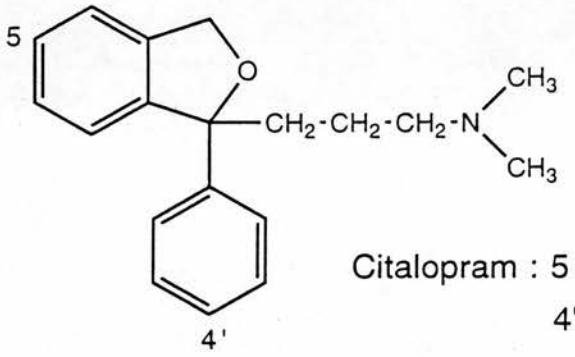
5-Azido-Citalopram (5-AC) : R = N_3

5-Iodo-Citalopram (5-IC) : R = I

Paroxetine

Fig. 3.1 Chemical structures of citalopram analogues and paroxetine

TABLE 3. 1 : Structure-Activity Relationships of Citalopram Analogues



Citalopram : 5 = CN
4' = F

5	4'		[^{14}C]5-HT uptake, IC_{50} (nM)
CN	F	(citalopram)	14
CN	Cl		17
CN	CN		29
Cl	F		21
F	F		34
Br	F		22
Cl	CN		29

Rabbit platelet-rich plasma was incubated with [^{14}C]5-HT in the presence of test compounds. Platelets were isolated by centrifugation, and residual radioactivity determined by liquid scintillation counting (taken from Bigler et al., 1977).

The purpose of the study in this chapter was to evaluate 5-AC as a photoaffinity ligand and 5-IC as a SPET ligand for the 5-HT transporter.

3.2 METHODS

3.2.1 Preparation of Rat Cortical Synaptosomes

The cerebral cortex from male Sprague Dawley rats (240-310 g) was dissected and gently homogenised in 20 vols (V/W) of ice cold 0.32 M sucrose using a glass teflon homogeniser, and centrifuged at 1,000g at 4°C for 10 min. The pellet was discarded and the supernatant was centrifuged at 17,000g at 4°C for 20 min. The crude synaptosomal pellet (P_2) was suspended in a modified Krebs-Ringer-phosphate buffer, pH 7.4 (122 mM NaCl, 4.82 mM KCl, 0.97 mM $CaCl_2$, 1.21 mM $MgSO_4$, 12.7 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 10 mM glucose and 1 mM ascorbic acid). For experiments to determine appropriate incubation time and tissue concentration for [^{3}H]5-HT uptake, the P_2 pellet was resuspended in 20 or 80 vols, and for experiments to examine drug potency for inhibition of [^{3}H]5-HT uptake 20 vols were used.

3.2.2 Preparation of Rat Cortical Membranes

The cerebral cortex from male Sprague Dawley rats (250-330 g) was dissected and homogenised in 50 vols of ice cold 50 mM Tris-HCl buffer (pH 7.4) containing 120 mM NaCl and 5 mM KCl using a glass teflon homogeniser. The homogenate was centrifuged at 30,000g at 4°C for 10 min. The resultant pellet was washed by resuspension in 50 vols of Tris-HCl buffer and recentrifuged. The final crude membrane pellet was

resuspended in Tris-HCl buffer (25 vols) and stored at -20°C until required. On the day of [^{3}H]paroxetine and [^{3}H]citalopram binding experiments, the membrane suspension was thawed at room temperature then diluted to 300 vols of Tris-HCl buffer.

3.2.3 [^{3}H]5-HT Uptake

Uptake of [^{3}H]5-HT into rat cortical synaptosomes was carried out by preincubating 100 µl aliquots of synaptosomal suspension (1.25 or 5 mg wet weight tissue/ml for experiments to determine appropriate incubation time and tissue concentration, 1.25 mg wet weight tissue/ml for experiments to examine drug potency) with Krebs-Ringer-phosphate buffer (control) or 6-7 concentrations of test drug in duplicate for 5 min at 37°C before addition of [^{3}H]5-HT (specific activity : 12 Ci/mmol, final concentration 5 nM) to give a final assay volume of 500 µl. Samples were vortexed briefly then incubated at 37°C for various times. Uptake was terminated by dilution with ice cold Krebs buffer (2 ml) and rapid filtration (Whatman GF/B filter) followed by two washes with 2 ml ice cold Krebs buffer. This procedure was carried out within 15 sec. In order to define the active component of [^{3}H]5-HT uptake, for assessment of the trapping of [^{3}H]5-HT, replicate zero-time samples (samples incubated in the absence of [^{3}H]5-HT were diluted with ice cold buffer prior to addition of [^{3}H]5-HT and then filtered) were carried out. For assessment of the trapping plus passive diffusion of [^{3}H]5-HT, incubation of samples at 37°C was carried out in the presence of 1 µM citalopram. Both gave the same results (data not shown). Therefore replicate zero-time samples were routinely used to

define the active component of [^{3}H]5-HT uptake. The filters were transferred to scintillation vials, then 100 µl of formic acid (100%) was added to each vial to digest the membrane protein followed 10 min later by addition of 4 ml of scintillation fluid (Packard Emulsifier Safe). The radioactivity in the vials was measured in a Packard 1900CA liquid scintillation analyser using automatic quench correction. Quadruplicate 100 µl samples of [^{3}H]ligand as standards were counted in order for the exact ligand concentration to be calculated for individual experiments.

3.2.4 [^{3}H]Paroxetine and [^{3}H]Citalopram Binding

Binding of [^{3}H]paroxetine and [^{3}H]citalopram to rat cortical membranes was carried out according to the methods of Habert et al. (1985) and D'Amato et al. (1987a), respectively. Binding of both radioligands was carried out by preincubating duplicate 0.5 ml aliquots of membrane suspension with 50 mM Tris-HCl containing 120 mM NaCl and 5 mM KCl (Tris-HCl buffer), pH 7.4 (control) or 10 concentrations of test drug in duplicate for 2 min at 25°C before addition of [^{3}H]paroxetine (specific activity : 29 Ci/mmol, final concentration 0.2 nM) or [^{3}H]citalopram (specific activity : 87 Ci/mmol, final concentration 0.5 nM) to give a final assay volume of 2 ml for [^{3}H]paroxetine binding or 1 ml for [^{3}H]citalopram binding. Samples were vortexed briefly then incubated at 25°C for various times. 10 µM unlabelled citalopram was used to define specific binding for both radioligands. Incubation was terminated at room temperature by rapid filtration under vacuum on a Brandel Cell Harvester using Whatman GF/B filters, pretreated with 0.05%

polyethyleneimine for 60 min, then washed twice with 5 ml Tris-HCl buffer. The filters were transferred to scintillation vials, then 100 µl of formic acid (100%) was added to each vial to digest the membrane protein followed 10 min later by addition of 4 ml of scintillation fluid (Packard Emulsifier Safe). The radioactivity in the vials was measured in a Packard 1900CA liquid scintillation analyser using automatic quench correction. Quadruplicate 100 µl samples of [^{3}H]ligand as standards were counted in order for the exact ligand concentration to be calculated for individual experiments.

3.2.5 Photoinactivation of The 5-HT Transporter by 5-Azido-Citalopram (5-AC)

A flow diagram for the photoinactivation experiments is shown in fig.3.2. Rat cortical membranes were prepared as described in section 3.2.2. Membrane suspensions (50 mg wet weight/5 ml) in 50 mM Tris-HCl buffer containing 120 mM NaCl and 5 mM KCl (pH 7.4) were incubated in the dark in the absence or presence of 5-AC (final concentration 200 nM) at 25°C for 30 min to reach equilibrium binding. After the 30 min incubation, for photolysis, the membrane suspensions (50 mg wet weight/5 ml) were contained in plastic petri dishes (60 x 15 mm) 2 cm (for 400 W irradiation) or 12 cm (for 15 W irradiation) below a U.V. lamp and irradiated for various times. Following irradiation, the membrane suspensions were transferred to centrifuge tubes, the petri dishes washed with 3 x 5 ml Tris-HCl buffer then tubes were centrifuged at 30,000g at 4°C for 10 min. The membrane pellets were resuspended in 20 ml of Tris-HCl buffer, incubated for 10 min at 25°C to allow

1) Suspension of rat cortical membranes
(50 mg wet weight/5 ml)

↓ 1. Incubate with 5-AC (200 nM) in the dark
25℃, 30 min

2) Irradiation with U.V. light (400 W or 15 W)

↓ 1. Add 5-AC (200 nM)

2. Incubate (25℃, 30 min)

3) Irradiation with U.V. light

↓

4) Wash membrane free of 5-AC

↓ 1. 30,000 g, 4℃, 10 min

2. Resuspend in buffer

3. Incubate (25℃, 10 min)

4. 30,000 g, 4℃, 10 min

5. Resuspend in buffer

6. Incubate (25℃, 10 min)

7. 30,000 g, 4℃, 10 min

8. Resuspend in buffer

5) Determine specific binding for [^{3}H]citalopram binding and compare with control membrane suspension

Fig.3.2 Flow diagram for photoinactivation of the 5-HT transporter by 5-AC. For single irradiation, procedure 2) was omitted.

reversibly bound drug to dissociate, then recentrifuged in order to remove unbound 5-AC and its photoactivated products. This washing and incubation procedure was repeated one additional time. Finally, pellets were resuspended in 15 ml of Tris-HCl buffer (final volumes 20 ml) and used for [^{3}H]citalopram binding. Membranes were incubated with [^{3}H]citalopram (0.5 nM) in the presence of 10 concentrations of unlabelled citalopram at 25°C for 60 min as described in section 3.2.4. For experiments using two U.V. steps, a second dose of 5-AC (final concentration 200 nM) was added to the membrane suspension immediately after the first irradiation, and the incubation continued for a further 30 min at 25°C followed by a second U.V. irradiation. Following the second irradiation, the washing and incubation steps were carried out as described above.

3.2.6 In Vitro [^{125}I]5-Iodo-citalopram (5-IC) Binding

Rat cortical membranes were prepared as described in section 3.2.2. The binding assays using appropriate amounts of [^{125}I]5-IC (2000 Ci/mmol, final concentration 0.05 nM) and a range of unlabelled 5-IC concentrations were carried out as described in section 3.2.4 ([^{3}H]citalopram binding). After filtration, the filter discs were transferred to Sterilin RT-30 plastic tubes and bound [^{125}I]5-IC determined using gamma spectrometry. 10 µM unlabelled citalopram was used to define specific binding. Quadruplicate 100 µl samples of [^{125}I]5-IC as standards were counted in order for the exact ligand concentration to be calculated for individual experiments.

3.2.7 Protein Assay

Protein was assayed according to Bradford (1976). 100 ml of 85% (w/v) phosphoric acid was added to Coomassie Brilliant Blue (100 mg) in 50 ml of 95% ethanol then diluted to a final volume of 1litre and filtered through Whatman Number 1 filter paper. This reagent (2.5 ml) was added to duplicate 100 µl of sample in the plastic cuvettes. The optical density of sample was read 20 min later at 595 nm on a spectrophotometer. Bovine serum albumin (25-200 µg/ml) dissolved in 50 mM Tris-HCl buffer was used as standard. The protein concentration of membrane samples was calculated from the standard curve. An example of the standard curve is shown in fig.3.3. Samples outwith the linear part of the standard curve were diluted with Tris buffer and re-assayed.

3.2.8 Data Analysis of Uptake and Binding Experiments

Data from individual experiments for [^{3}H]5-HT uptake, [^{3}H]paroxetine and [^{3}H]citalopram binding were analysed by least squares fit to the logistic expression :

$$Y = MX^P / (X^P + IC_{50})$$

and K_D or Km values calculated. Ki values were calculated from the equation :

$$Ki = IC_{50} / (1 + L/K_D \text{ or } Km)$$

where L is ligand concentration. Bmax values were calculated from the equation :

$$b = Bmax \cdot L / (L + K_D)$$

where b is bound ligand concentration at the [^{3}H]ligand concentration L. Vmax values were calculated from the equation :

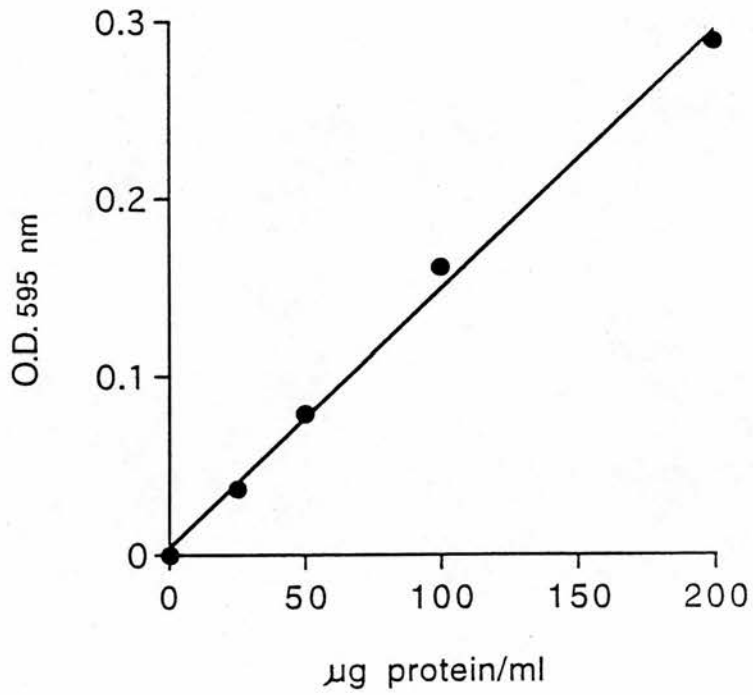


Fig. 3.3 An example of a typical protein standard curve. The curve was constructed over the range 25 - 200 µg bovine serum albumin/ml, and is essentially linear over this range. Protein concentration of membrane samples was calculated from the standard curve.

$$v = Vmax \cdot S / (S + Km)$$

where S is [^{3}H]5-HT substrate concentration. The values for K_D, Km, Ki, Bmax and Vmax were expressed as mean ± S.E.M. from three to nine independent experiments.

3.2.9 In Vivo Distribution of [^{125}I]5-IC

Male Sprague Dawley rats (220-330 g) were allowed access to food and water ad libitum prior to the experiments. Under urethane anaesthesia (10% solution, 10 ml/kg, i.p.), the left femoral vein was catheterized with polyethylene tubing for drug administration. Rats were injected over 30 sec with 20 µCi of [^{125}I]5-IC (corresponding to 20 nmol of [^{125}I]5-IC) in 250 µl of saline containing 10% ethanol, 1% Tween-80 and 10 units of heparin through the cannula placed in the left femoral vein. To complete injection of the drug, 500 µl of the heparinized saline was used to flush the cannula immediately following infusion of the [^{125}I]5-IC. For experiments to determine the time course of [^{125}I]5-IC distribution, at various times post-injection (30, 60, 90, 180 and 300 min), samples of blood (~5ml) were taken in a heparinised syringe from the inferior vena cava and the rats were killed by decapitation. The brains were removed, divided into 6 regions : rostral cortex, caudal cortex (cerebral cortex - rostral cortex), hippocampus, brainstem, midbrain and cerebellum. In addition, liver, kidney, heart, lung and thyroid were removed, blotted dry and weighed. Samples 100 to 200 mg of each organ were taken, again blotted to remove as much blood or urine as possible and transferred to vials and radioactivity determined using gamma spectrometry. For the 60 min and 300 min rats, the right femoral artery was

also cannulated to obtain arterial blood samples at various times post-injection of [^{125}I]5-IC. At each time point the first 100 µl of blood was discarded since it probably represented the dead volume in the cannula. A 300 µl sample was then collected in a heparinised microcentrifuge tube. A 100 µl sample was transferred to vials and radioactivity determined.

In order to assess 5-HT transporter bound [^{125}I]5-IC, heparinised saline or paroxetine (1 mg/kg) was injected via the left femoral vein 5 min before injection of [^{125}I]5-IC. The rats were killed 2 hr after [^{125}I]5-IC injection, and brain samples prepared as described above. The results for the tissue distribution study were expressed as % injected dose per gram of tissue (%D/g) or amol per milligram of tissue (amol/mg), and results for the blood distribution study were expressed as % injected dose per ml of blood (%D/ml). In order to calculate the exact amount of [^{125}I]5-IC injected into the rats, standards were obtained by counting quadruplicate 100 µl samples of a 1 : 500 dilution of original radioactivity used for injection.

3.2.10 Pharmacokinetic Analysis

The data for radioactivity in blood expressed as %D/ml after [^{125}I]5-IC injection were fitted to biexponential functions using least squares regression analysis since the fitting to biexponential functions was better than monoexponential functions. The equation used was $Y = A \times e^{(-\alpha t)} + B \times e^{(-\beta t)}$, where t is time after administration, Y is the measured radioactivity, A and B are the coefficients of the exponential terms, α is the apparent distribution rate constant, β is the

apparent terminal rate constant. The data for radioactivity in brain and peripheral tissues were fitted to monoexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-Ke \cdot t)}$, where Ke is the elimination rate constant. The following pharmacokinetic parameters were calculated : 1) half-life ($t_{1/2}$) = 0.693/Ke or α or β, 2) the area under the radioactivity concentration-time curve from zero to time infinity (AUC). AUC was calculated using the trapezoid method.

3.2.11 Statistical Analysis

The Student's t-test was used for statistical comparison of data of in vitro binding and in vivo binding between drugs or conditions when necessary. Differences were considered significant when P was < 0.05.

3.2.12 Materials

5-AC and 5-IC were synthesised in the Department of Pharmacology, University of Edinburgh by Dr.I.M.Dawson. 5-AC was dissolved in ethanol, the bottle wrapped in tin foil, and stored at 4°C in the dark. 5-IC was dissolved in glass distilled water. [^{125}I]5-IC was synthesised and purified by Dr.I.M.Dawson in the Department of Pharmacology, University of Edinburgh. [^{125}I]5-IC was stored at -20°C until required. [^{3}H]5-HT creatinine sulphate (specific activity : 12 Ci/mmol) and [^{3}H]citalopram (specific activity : 87 Ci/mmol) were obtained from Amersham International. [^{3}H]Paroxetine (specific activity : 29 Ci/mmol) was obtained from NEN. These radioligands were diluted to 1 μM in glass distilled water and stored under liquid

nitrogen in volumes sufficient for individual experiments. The following drugs were donated as gifts : citalopram (SmithKline Beecham), paroxetine (Ferrosan) and fluoxetine (Eli Lilly). Other drugs and reagents were obtained from Sigma.

3.3 RESULTS

Initial experiments were carried out to establish and validate the incubation conditions required to measure active uptake of [^{3}H]5-HT and specific binding of [^{3}H]citalopram and [^{3}H]paroxetine.

3.3.1 Kinetics and Pharmacology of [^{3}H]5-HT Uptake into Rat Cortical Synaptosomes

The time course of active [^{3}H]5-HT (5 nM) uptake into rat cortical synaptosomes (5 mg or 1.25 mg wet weight tissue/ml) was linear for only a short time (Fig.3.4). Therefore, in order to measure approximate initial rates of 5-HT uptake to ensure an accurate assessment of drug potency, incubations of [^{3}H]5-HT with synaptosomes were limited to 5 min. At this time point, active uptake represented about 70% of total uptake. In the 5 min incubation period, 17% (5 mg wet weight tissue/ml) and 7% (1.25 mg wet weight tissue/ml) of the total content of [^{3}H]5-HT was taken up into synaptosomes. When drugs were examined using this assay, their potencies were calculated using the equation :

$Ki = IC_{50} / (1 + [S]/Km)$ ([S] : substrate concentration).

However, the equation is valid only when certain conditions are satisfied i.e. $[S]t \approx [S]a \approx [S]p$ ([S]t : total substrate concentration, [S]a : ligand concentration in the absence of

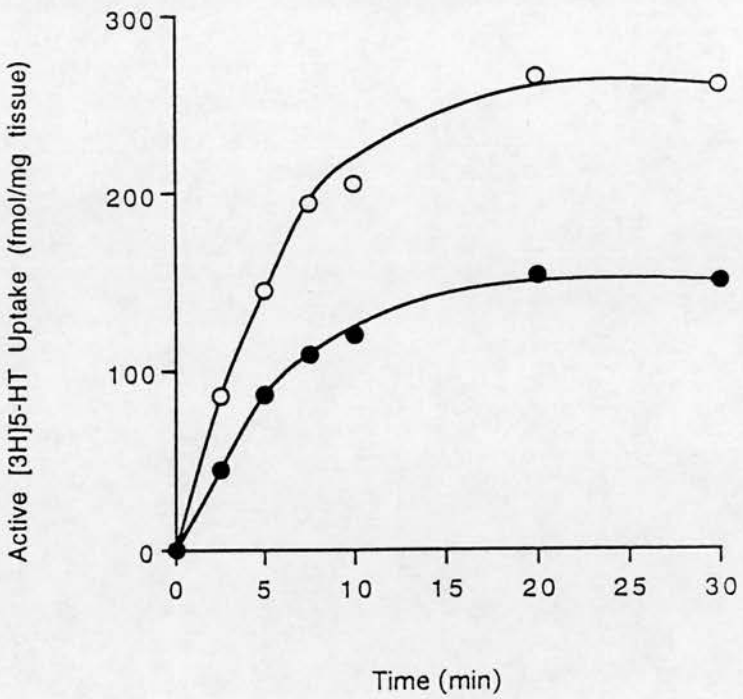


Fig. 3.4 Time course of [^{3}H]5-HT uptake into rat cortical synaptosomes. Synaptosomes (1.25 or 5 mg wet weight tissue / tube) were incubated with [^{3}H]5-HT (5 nM) for various times at 37°C. Replicate zero-time samples were carried out to define the active component of [^{3}H]5-HT uptake. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ○ 5 mg tissue; ● 1.25 mg tissue.

inhibitor, [S]p : ligand concentration in the presence of inhibitor). Using 5 mg wet weight tissue/ml of synaptosomes, [S]a was 4.2 nM since 17% of [^{3}H]5-HT (5 nM) was taken up into synaptosomes whereas [S]p was 5 nM when test drug completely inhibited [^{3}H]5-HT uptake. When 1.25 mg wet weight tissue/ml was used, [S]a is 4.7 nM. Thus, when the latter tissue concentration of synaptosomes was used for [^{3}H]5-HT uptake experiments, substrate depletion during the incubation was essentially absent and so allowed accurate Ki values to be determined. Therefore, a tissue concentration of 1.25 mg wet weight tissue/ml was routinely used when the potency of various drugs as inhibitors of [^{3}H]5-HT uptake into synaptosomes were determined. Analysis of [^{3}H]5-HT uptake data using unlabelled 5-HT from three separate experiments gave a Km value of 24.1 ± 3.2 nM and a Vmax value of 0.80 ± 0.07 pmol/5min/mg wet weight tissue. These values correspond well with data shown by Richelson and Pfenning (1984), Marcusson et al. (1986) and Thomas et al. (1987).

The pharmacology of [^{3}H]5-HT uptake into rat cortical synaptosomes was examined using a range of uptake inhibitors (Table 3.2, Fig.3.5a). The selective 5-HT uptake inhibitors, paroxetine (Ki = 0.83 ± 0.05 nM), citalopram (Ki = 2.0 ± 0.4 nM) and fluoxetine (Ki = 11.3 ± 2.0 nM) had high affinity for the 5-HT uptake sites whereas the tricyclic antidepressant, desipramine gave a Ki value of 156 ± 11 nM. The Ki value of each compound for [^{3}H]5-HT uptake was consistent with the known pharmacology of the amine transport in serotonergic neurons (Hyttel, 1982; Thomas et al, 1987). Thus the most potent inhibitor was paroxetine whereas the least potent inhibitor was

TABLE 3.2 : Pharmacology of 5-HT Transporter

	Km/K_D/Ki (nM)		
	[^{3}H]5-HT uptake	[^{3}H]Paroxetine binding	[^{3}H]Citalopram binding
5-HT	24.1 ± 3.2 (3)	836 ± 72 (4)	349 ± 35 (4)
Citalopram	2.0 ± 0.4 (9)	2.1 ± 0.2 (9)	0.94 ± 0.24 (7)
Paroxetine	0.83 ± 0.05 (3)	0.20 ± 0.02 (6)	0.12 ± 0.01 (7)
Fluoxetine	11.3 ± 2.0 (3)	6.1 ± 1.2 (6)	6.3 ± 1.1 (6)
Desipramine	156 ± 11 (3)	297 ± 49 (6)	154 ± 11 (5)

Synaptosomes were incubated with [^{3}H]5-HT (5 nM) in the absence or presence of increasing concentrations of test drug for 5 min at 37°C. Replicate zero-time samples were carried out to define the active component of [^{3}H]5-HT uptake. Membranes were incubated with [^{3}H]paroxetine (0.2 nM) or [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of test drug for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Ki values were calculated from the equation Ki = IC_{50} / (1+ L/K_D or Km). Values are the mean ± S.E.M. from the number of separate experiments shown in parentheses.

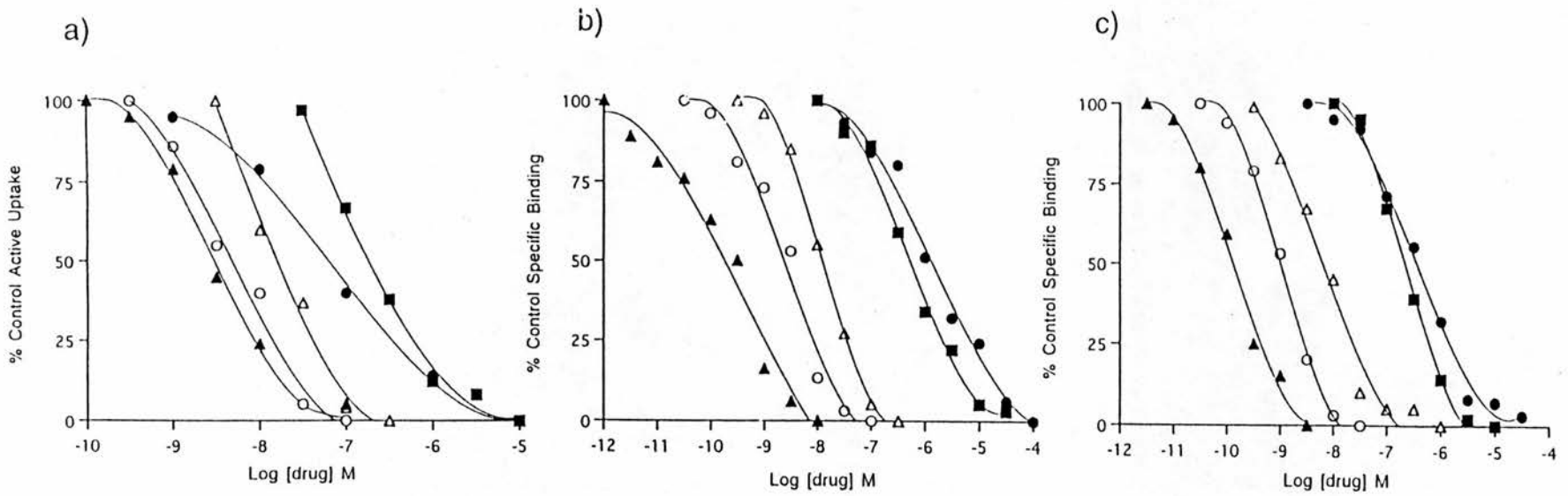


Fig. 3.5 Inhibition of a) [^{3}H]5-HT uptake, b) [^{3}H]paroxetine binding and c) [^{3}H]citalopram binding. Synaptosomes were incubated with [^{3}H]5-HT (5 nM) in the absence or presence of increasing concentrations of test drug for 5 min at 37°C. Replicate zero-time samples were carried out to define active component of [^{3}H]5-HT uptake. Membranes were incubated with [^{3}H]paroxetine (0.2 nM) or [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of test drug for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● 5-HT; ○ Citalopram; ▲ Paroxetine; △ Fluoxetine; ■ Desipramine.

desipramine which is known to be a noradrenaline uptake inhibitor rather than a 5-HT uptake inhibitor (Hyttel, 1982; Richelson and Pfenning, 1984).

3.3.2 Kinetics and Pharmacology of [^{3}H]Paroxetine Binding to Rat Cortical Membranes

The time course of specific binding of [^{3}H]paroxetine (0.2 nM) to rat cortical membranes at 25°C reached equilibrium after approximately 30 min incubation (Fig.3.6). In order to ensure that equilibrium was attained, an incubation time of 60 min was used for further investigation of [^{3}H]paroxetine binding. At this concentration, specific binding represented about 82% of total binding. Specific [^{3}H]paroxetine binding to rat cortical membranes was shown to be linear with tissue concentration from at least 25 to 70 µg of protein per 2 ml assay (Fig.3.7). A final assay volume of 2 ml was used for the [^{3}H]paroxetine binding assay in order to prevent depletion of the free ligand concentration. Therefore, protein concentrations within this range were used for all subsequent experiments. Analysis of [^{3}H]paroxetine binding data using unlabelled paroxetine from six separate experiments gave a K_D value of 0.20 ± 0.02 nM and a Bmax value of 0.82 ± 0.08 pmol/mg protein. These values were consistent with data shown by Habert et al. (1985).

Inhibition of [^{3}H]paroxetine binding to rat cortical membranes by 5-HT, citalopram, paroxetine, fluoxetine and desipramine was tested (Table 3.2, Fig.3.5b). 5-HT inhibited [^{3}H]paroxetine binding to rat cortical membranes, giving a Ki value of 836 ± 72 nM. The selective 5-HT uptake inhibitors,

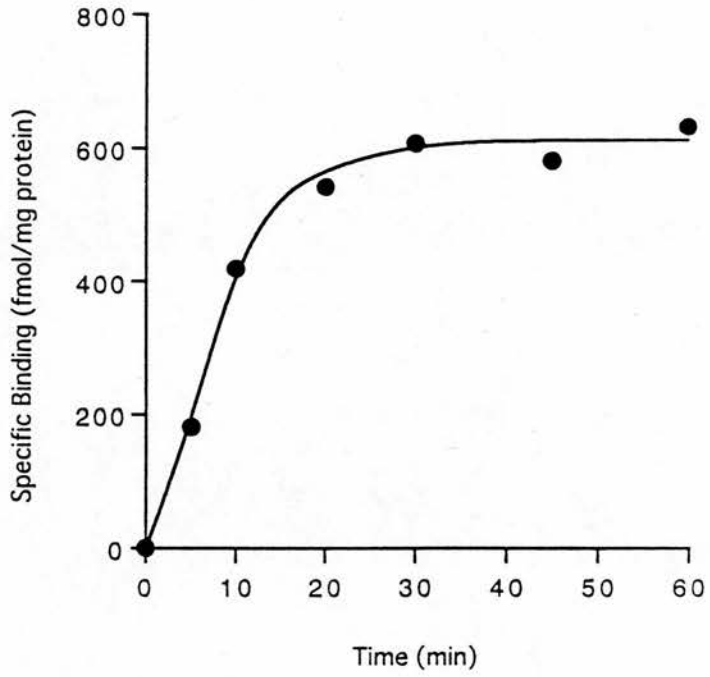


Fig. 3.6 Time course of [^{3}H]paroxetine binding to rat cortical membranes. Membranes were incubated with [^{3}H]paroxetine (0.2 nM) for various times at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations.

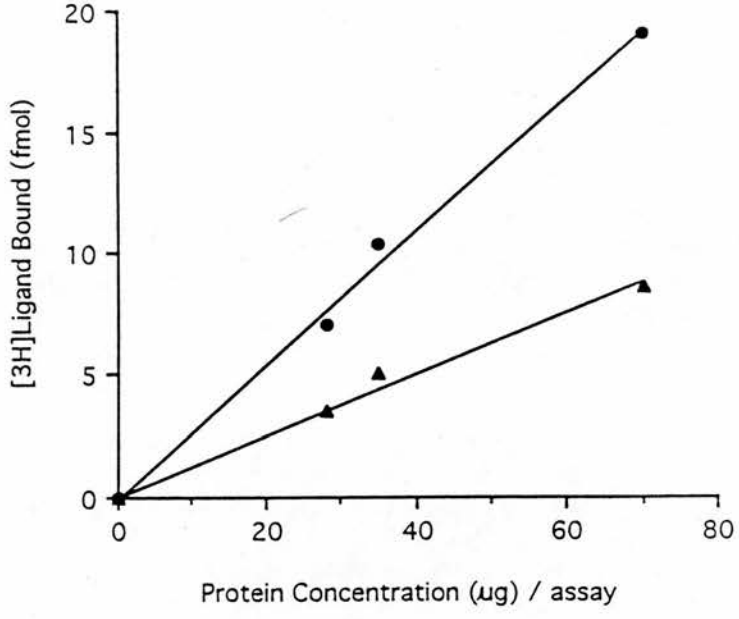


Fig. 3.7 Effect of protein concentration on [^{3}H]paroxetine and [^{3}H]citalopram binding. Membranes were incubated with [^{3}H]paroxetine (0.2 nM) or [^{3}H]citalopram (0.5 nM) for 60 min at 25°C as a final assay volume of 2 ml and 1 ml respectively. Non-specific binding was determined in the presence of 10 µM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● [^{3}H]Paloxetine; ▲ [^{3}H]Citalopram.

citalopram and fluoxetine had high affinity for [^{3}H]paroxetine binding sites with Ki values of 2.1 ± 0.2 nM and 6.1 ± 1.2 nM respectively, whereas desipramine (Ki = 297 ± 49 nM) showed affinity in the high nanomolar range. Of the drugs tested, paroxetine (K_D = 0.20 ± 0.02 nM) was the most potent inhibitor for [^{3}H]paroxetine binding followed by citalopram. Data were consistent with [^{3}H]paroxetine binding to the 5-HT transporter (Habert et al.,1985).

3.3.3 Kinetics and Pharmacology of [^{3}H]Citalopram Binding to Rat Cortical Membranes

The time course of [^{3}H]citalopram (0.5 nM) binding to rat cortical membranes at 25°C showed that equilibrium was attained after approximately 30 min incubation (Fig.3.8), similar to that found with [^{3}H]paroxetine binding. Therefore an incubation time of 60 min was used for further experiment of [^{3}H]citalopram binding. At this concentration, specific binding represented about 85% of total binding. Specific [^{3}H]citalopram binding was shown to be linearly related to protein concentration in the range 25-70 µg protein per 1 ml assay (Fig.3.7). As a final assay volume, 1 ml was used because there was virtually no free ligand depletion. Therefore, protein concentrations in this range were used for all subsequent experiments. Data analysis of [^{3}H]citalopram binding using unlabelled citalopram gave a K_D value of 0.94 ± 0.24 nM and a Bmax value of 0.85 ± 0.13 pmol/mg protein. These values were consistent with data shown by D'Amato et al. (1987a).

Inhibition of [^{3}H]citalopram binding to rat cortical membranes by the same drugs used for [^{3}H]5-HT uptake and

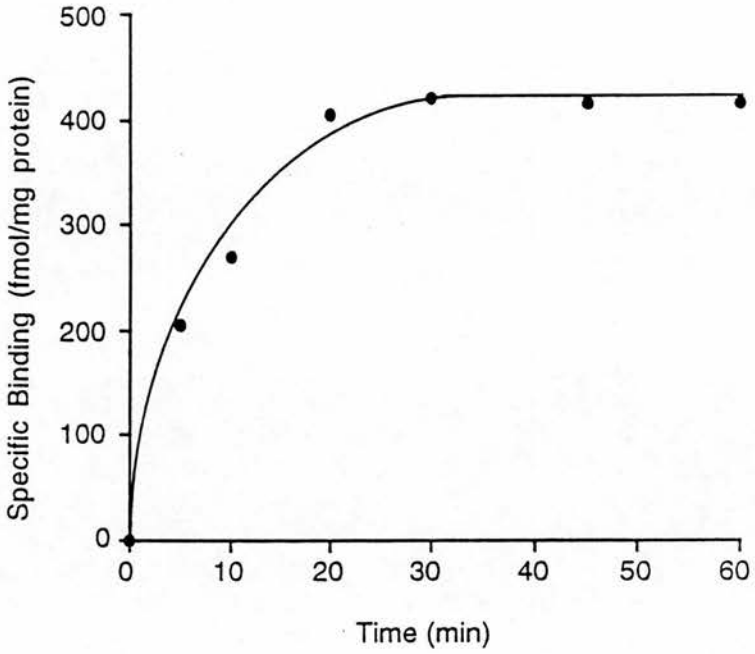


Fig. 3.8 Time course of [^{3}H]citalopram binding to rat cortical membranes. Membranes were incubated with [^{3}H]citalopram (0.5 nM) for various times at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations.

[^{3}H]paroxetine binding was examined (Table 3.2, Fig.3.5c). Paroxetine and fluoxetine inhibited the binding of [^{3}H]citalopram to rat brain membranes with a Ki value of 0.12 ± 0.01 nM and 6.3 ± 1.1 nM, respectively. As with [^{3}H]paroxetine binding, desipramine (Ki = 154 ±11 nM) and 5-HT (Ki = 349 ± 35 nM) were relatively weak inhibitors for [^{3}H]citalopram binding. Thus the most potent inhibitor for [^{3}H]citalopram binding was paroxetine which was approximately eight fold more potent than citalopram (K_D = 0.94 ± 0.24 nM).

3.3.4 Decomposition of 5-AC by U.V. Irradiation

5-AC was synthesised as a photoaffinity ligand for the 5-HT transporter. Prior to examining whether it binds irreversibly to the 5-HT transporter following U.V. irradiation, the required irradiation time for photo-decomposition was determined in the absence of brain membranes. Petri dishes containing 5 ml samples of 10 μM 5-AC in Tris-HCl buffer were exposed to U.V. light (400 W, wavelength 365 nm or 15 W, 254 nm) for various times then the amount of authentic 5-AC remaining was determined by HPLC (Table 3.3). After 1 min irradiation by U.V. light (400 W), 5-AC disappeared completely whereas 50% of 5-AC remained after irradiation for 30 sec. U.V. irradiation with 15 W produced an 81% and 98% reduction in the concentration of 5-AC after 30 sec and 1 min, respectively. 5-AC disappeared completely following 2 min irradiation.

TABLE 3.3 : Decomposition of 5-AC by U.V. Irradiation

Irradiation time (min)	5-AC concentration (% of control)	
	400 W	15 W
0.5	50	19.1
1	0	1.8
2		0

5-AC (10 μM) in Tris-HCl buffer (5 ml) in petri dishes 2 cm (400 W) or 12 cm (15 W) below a U.V. lamp (400 W, wavelength 365 nm or 15 W, wavelength 254 nm) was irradiated for various times and then its concentration was determined by HPLC (column : Ultrasphere ODS 5 μ, 4.6 mm x 15 cm, eluting solvent : 10% 0.1 M $NH_4^+OAc^-$/90% ethanol, detection 270 nm).

3.3.5 Effect of 5-AC and U.V. Irradiated 5-AC on [^{3}H]Citalopram Binding

It was important to determine the affinity of 5-AC for the 5-HT transporter. 5-AC was therefore tested as an inhibitor of [^{3}H]citalopram binding to rat cortical membranes (Table 3.4, Fig.3.9). 5-AC inhibited [^{3}H]citalopram binding to rat cortical membranes with a Ki value of 1.65 ± 0.14 nM which was only 1.8-fold less potent than citalopram, indicating that incorporation of an azido moiety at the 5-position of citalopram had virtually no effect on affinity for the 5-HT transporter. Similarly it was also important to determine the affinity of the U.V. irradiated product of 5-AC for the 5-HT transporter. Therefore the effect of 5-AC irradiated for 2 min (15 W) on [^{3}H]citalopram binding to rat cortical membranes was examined since as shown in table 3.3 virtually no authentic 5-AC remained under this condition. The decomposed 5-AC (UV-5-AC) inhibited [^{3}H]citalopram binding to rat cortical membranes with a Ki value of 14.25 ± 0.78 nM which was about 9 and 15 times less potent compared with 5-AC and citalopram, respectively (Table 3.4, Fig.3.9). Because of the lower affinity of UV-5-AC and thus a faster dissociation rate, removal of UV-5-AC which was not irreversibly bound to the 5-HT transporter would be easier and more efficient during the washing procedure compared with removal of 5-AC.

3.3.6 Membrane Irradiation with 400 W in the Presence of 5-AC

The [^{3}H]citalopram binding assay was used to assess whether 5-AC could be used as a photoaffinity ligand for the 5-HT transporter. If 5-AC bound irreversibly to the transporter

TABLE 3.4 : Effect of 5-Azido-citalopram (5-AC) and U.V. Irradiated 5-AC (UV-5-AC) on [^{3}H]Citalopram Binding to Rat Cortical Membranes

	K_D / Ki (nM) [^{3}H]Citalopram binding
5-AC	1.65 ± 0.14 (5)
UV-5-AC	14.25 ± 0.78 (3)
Citalopram	0.94 ± 0.24 (7)

Membranes were incubated with [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of test drug (10^{-11} M to 10^{-6} M, final concentrations) for 60 min at 25°C. UV-5-AC was obtained by U.V. irradiation (15W for 2 min) of 5-AC. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Ki values were calculated from the equation $Ki = IC_{50} / (1 + L/K_D)$. Values are the mean ± S.E.M. from the number of separate experiments shown in parentheses.

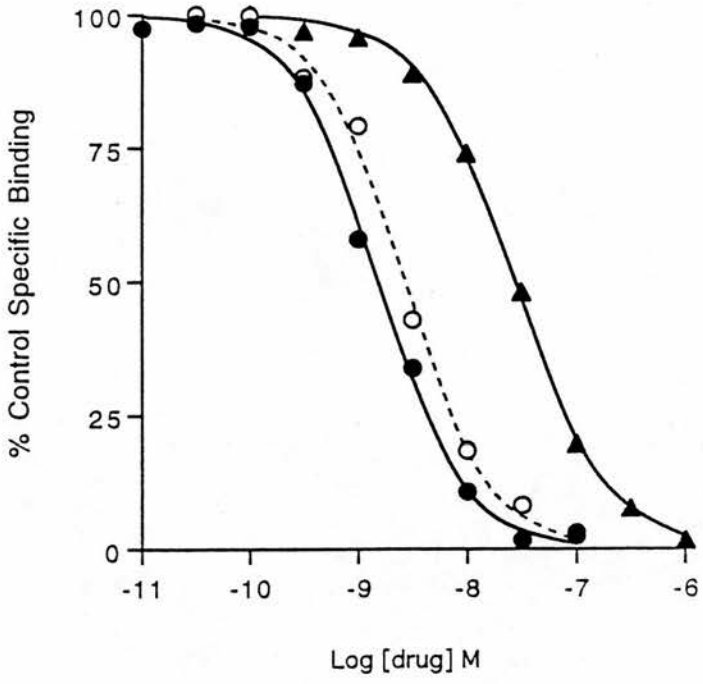


Fig. 3.9 Inhibition of [^{3}H]citalopram binding to rat cortical membranes by citalopram, 5-AC and UV-5-AC. Membranes were incubated with [^{3}H]citalopram (0.5nM) in the absence or presence of increasing concentrations of test drug for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● Citalopram; ○ 5-AC; ▲ UV-5-AC.

following U.V. exposure, a decrease in the density of binding sites for [^{3}H]citalopram (Bmax) would be predicted. Appropriate control samples were also carried out firstly to determine the direct effect of U.V. exposure on the brain membranes and secondly to determine the efficiency of washing the membranes free of 5-AC and UV-5-AC prior to [^{3}H]citalopram binding. Rat cortical membranes were incubated at 25°C for 30 min in the absence and presence of 5-AC in the dark, at a final concentration of 200 nM. This concentration of 5-AC is about 100 times its Ki value (Table 3.4) and is thus sufficient to occupy all the 5-HT transporters as shown by its ability to completely displace [^{3}H]citalopram binding to rat cortical membranes (Fig.3.9). After the 30 min incubation, membrane suspensions were U.V. irradiated with 400 W once (1 min) or twice (30 sec). Following extensive washing of the membranes to remove the 5-AC and UV-5-AC, [^{3}H]citalopram binding was carried out in the presence of a range of concentration of unlabelled citalopram in order to determine K_D and Bmax values.

a) Single Irradiation with 400 W (Table 3.5)

In control membranes (non-irradiated in the absence of 5-AC), the K_D and Bmax values for [^{3}H]citalopram binding were 1.15 ± 0.43 nM and 1031.6 ± 72.7 fmol/mg protein, respectively. In the non-irradiated membranes in the presence of 5-AC, the K_D (1.07 ± 0.13 nM) and Bmax (978.4 ± 48.6 fmol/mg protein) were not significantly different from those for control membranes. The K_D values for [^{3}H]citalopram indicate that 5-AC and UV-5-AC were removed from the membranes by the washing procedure prior to the binding assay being carried out. U.V. irradiation for

TABLE 3.5 : Effect of Single U.V. Irradiation (400 W) on [^{3}H]Citalopram Binding

Treatment			Bmax	K_D
U.V.	5-AC	N	(fmol/mg protein)	(nM)
-	- (control)	4	1031.6 ± 72.7	1.15 ± 0.43
-	+	4	978.4 ± 48.6	1.07 ± 0.13
1 min	-	3	979.2 ± 46.6	0.94 ± 0.25
1 min	+	4	870.2 ± 93.0	1.23 ± 0.27

Membranes were incubated in the absence (-) or presence (+) of 5-AC (200 nM) in the dark for 30 min at 25°C and then irradiated with 400 W for 1 min (- ; non-irradiation). After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. IC_{50} values were obtained from analysis of the inhibition curves from the equation $Y = MX^P / (X^P + IC_{50})$ and K_D values calculated ($K_D = IC_{50}$ - [^{3}H]citalopram). Bmax values were determined from the equation $b = Bmax\ L / (L + K_D)$. Values are the mean ± S.E.M. from the number of separate experiments shown in table (N).

1 min in the absence of 5-AC did not produce an alteration in Bmax (979.2 ± 46.6 fmol/mg protein) or K_D (0.94 ± 0.25 nM) compared with those for control membranes. This result suggests that U.V. irradiation for 1 min did not directly affect the 5-HT transporters. When compared with the irradiated membranes in the absence of 5-AC, there was no significant difference in the Bmax (870.2 ± 93.0 fmol/mg protein) or K_D (1.23 ± 0.27 nM) for the irradiated membranes in the presence of 5-AC. In addition, as shown in fig.3.10, there was no significant difference in specific [^{3}H]citalopram binding at the ligand concentration of 0.5 nM for the irradiated membranes in the presence of 5-AC compared with the irradiated membranes in the absence of 5-AC. These results indicate that 5-AC cannot produce a covalent bond at the [^{3}H]citalopram binding sites with a single irradiation with 400 W.

b) Repeated Irradiation with 400 W (Table 3.6)

Since no significant change in Bmax was observed with a single U.V. irradiation, indicating that it was not clear whether 5-AC binds irreversibly to the transporter it was decided to use repeated irradiation. For these experiments, thirty seconds was chosen as the irradiation time in order to avoid overheating the membrane preparations with the U.V. lamp because repeated irradiation (1 min x 2) in the absence of 5-AC produced a significant reduction (about 60%) in [^{3}H]citalopram binding in preliminary experiments (data not shown). Using two 30 sec irradiations the Bmax (745.7 ± 96.8 fmol/mg protein) and K_D (0.46 ± 0.12 nM) of [^{3}H]citalopram binding for irradiated

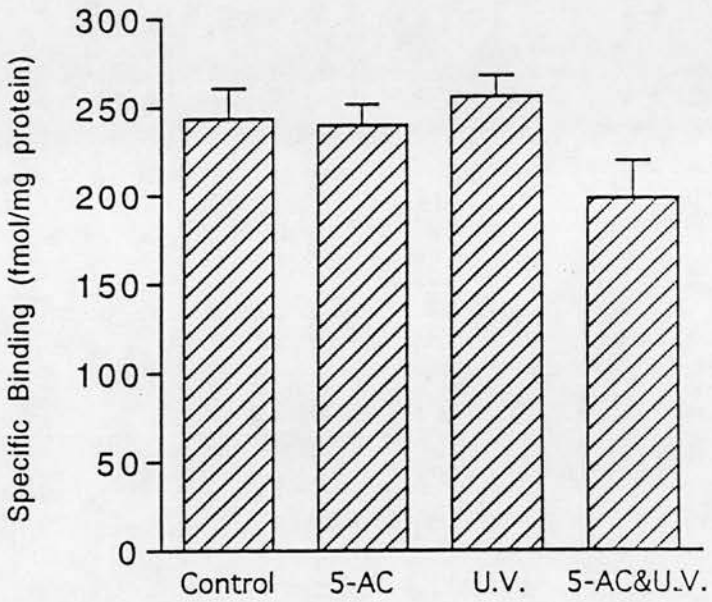


Fig. 3.10 Effect of single U.V. irradiation (400 W) on specific binding of [^{3}H]citalopram at 0.5 nM. Rat cortical membranes were incubated in the absence or presence of 5-AC (200 nM) in the dark for 30 min at 25°C and then irradiated with 400 W for 1 min. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) for 60 min at 25°C in the absence or presence of 10 µM unlabelled citalopram. Specific binding at 0.5 nM (fmol / mg protein) was calculated from binding data. Data are the mean ± S.E.M. from 3 or 4 separate experiments. Control : non-irradiated membranes in the absence of 5-AC; 5AC : non-irradiated membranes in the presence of 5-AC; U.V. : irradiated membranes in the absence of 5-AC; 5-AC & U.V. : irradiated membranes in the presence of 5-AC.

TABLE 3.6 : Effect of Repeated U.V. Irradiation (400W) on [^{3}H]Citalopram Binding

Treatment				Bmax	K_D
U.V.	5-AC		N	(fmol/mg protein)	(nM)
-	-	(control)	6	848.6 ± 51.0	0.76 ± 0.09
-	+		5	808.4 ± 69.4	1.42 ± 0.29 *
30 sec	-		5	745.7 ± 96.8	0.46 ± 0.12
30 sec	+		6	N.D.	N.D.

Membranes were incubated in the absence (-) or presence (+) of 5-AC (200 nM) in the dark for 30 min at 25°C then irradiated with 400 W for 30 sec (- ; non-irradiation). A second dose of 5-AC (200 nM) was added and the incubation and irradiation repeated. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Bmax and K_D values were determined as described in table 3.5. Values are the mean ± S.E.M. from the number of separate experiments shown in table (N). * ; $P < 0.05$ compared with control. N.D. ; calculation of Bmax and K_D value was not possible.

membranes in the absence of 5-AC were not significantly different from those for control membranes (Bmax = 848.6 ± 51.0 fmol/mg protein, K_D = 0.76 ± 0.09 nM). Thus repeated irradiation (30 sec x 2) did not directly affect the membranes. In the non-irradiated membranes in the presence of 5-AC, the Bmax (808.4 ± 69.4 fmol/mg protein) was not different from that for control membranes, but a significant difference in K_D value between the two membrane preparations was observed (control : 0.76 ± 0.09 nM, 5-AC : 1.42 ± 0.29 nM, $P < 0.05$ vs control). These results may indicate that 5-AC when added twice at 200 nM was not completely removed from the membrane suspensions even with extensive washing. For this change in apparent K_D value it can be calculated that about 1 nM 5-AC would have been required to remain in the final membrane suspension. Since UV-5-AC has about 9 fold lower affinity than 5-AC, 1 nM UV-5-AC remaining in the irradiated membranes would have had virtually no inhibitory effect.

In irradiated membranes in the presence of 5-AC, it was not possible to analyse the inhibition curves for citalopram accurately using a logistic fit and thus calculate the K_D and Bmax values for [^{3}H]citalopram binding because of the small amounts of specific binding obtained in some experiments. Therefore the specific [^{3}H]citalopram binding at the ligand concentration of 0.5 nM was directly compared with those for other membranes (Table 3.7, Fig.3.11). The specific binding (176.0 ± 17.3 fmol/mg protein) for non-irradiated membranes in the presence of 5-AC was significantly smaller than that for control membranes ($P < 0.05$ vs control), and as described earlier it is presumably due to incomplete removal of 5-AC

TABLE 3.7 : Effect of Repeated U.V. Irradiation (400W) on Specific Binding of [^{3}H]Citalopram at 0.5 nM

Treatment				Specific Binding
U.V.	5-AC		N	(fmol/mg protein)
-	-	(control)	6	247.8 ± 17.4
-	+		5	176.0 ± 17.3 *
30 sec	-		5	254.2 ± 21.2
30 sec	+		6	65.2 ± 18.8 **, #

Membranes were incubated in the absence (-) or presence (+) of 5-AC (200 nM) in the dark for 30 min at 25°C then irradiated with 400 W for 30 sec (- ; non-irradiation). A second dose of 5-AC (200 nM) was added and the incubation and irradiation repeated. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) for 60 min at 25°C in the absence or presence of 10 μM unlabelled citalopram to determine specific binding. Values are the mean ± S.E.M. from the number of separate experiments shown in table (N). * ; $P < 0.05$, ** $P < 0.001$ compared with control. # ; $P < 0.01$ compared with non-irradiation in the presence of 5-AC (U.V. - ; 5-AC +).

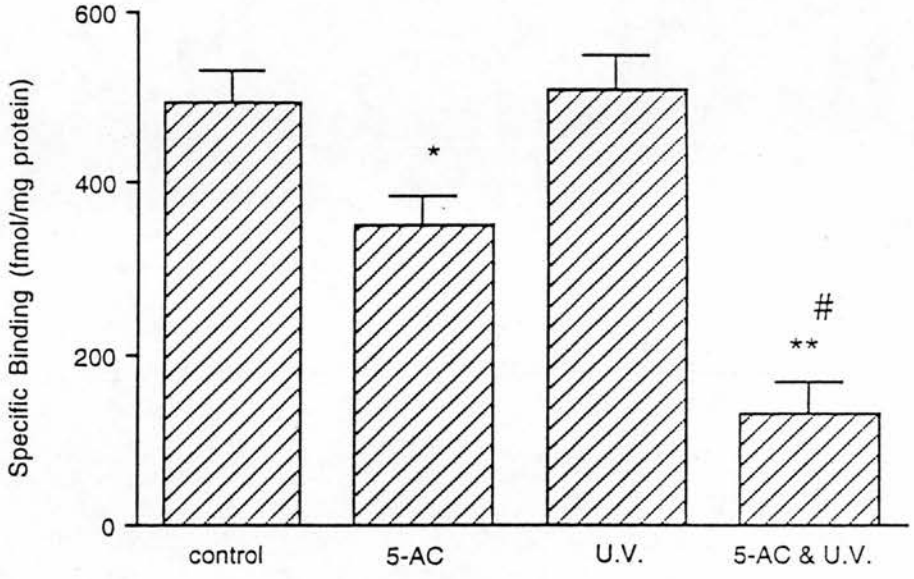


Fig. 3.11 Effect of repeated U.V. irradiation (400 W) on specific binding of [^{3}H]citalopram at 0.5 nM. Rat cortical membranes were incubated in the absence or presence of 5-AC (200 nM) in the dark for 30 min at 25°C and then irradiated with 400 W for 30 sec. A second dose of 5-AC (200 nM) was added and the incubation and irradiation repeated. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) for 60 min at 25°C in the absence or presence of 10 μM unlabelled citalopram. Specific binding at 0.5 nM (fmol / mg protein) was calculated from binding data. Values are the mean ± S.E.M. from 5 or 6 separate experiments. control : non-irradiated membranes in the absence of 5-AC; 5-AC : non-irradiated membranes in the presence of 5-AC; U.V. : irradiated membranes in the absence of 5-AC; 5-AC & U.V. : irradiated membranes in the presence of 5-AC. * ; $P < 0.05$, ** $P < 0.001$ compared with control. # ; $P < 0.01$ compared with 5-AC.

during the 3 washing cycles. In the presence of 5-AC, repeated irradiation produced a significant 74% reduction ($P < 0.001$) in specific binding (65.2 ± 18.8 fmol/mg protein) when compared with that for control membranes (247.8 ± 17.4 fmol/mg protein) or irradiated membranes in the absence of 5-AC (254.2 ± 21.2 fmol/mg protein). Following the washing procedure, since UV-5-AC would not have been present at a sufficiently high concentration to cause inhibition, the reduction in binding would be due to a decrease in the Bmax, suggesting that 5-AC binds irreversibly to about 70% of the 5-HT transporter following two 30 sec irradiation exposures.

3.3.7 Membrane Irradiation with 15 W in the Presence of 5-AC

Rat cortical membranes were incubated at 25°C for 30 min in the absence and presence of 5-AC (200 nM) or UV-5-AC (200 nM) in the dark. After incubation, membrane suspensions were U.V. irradiated with 15 W once (1, 2 or 4 min) or twice (2 min) then the membranes were washed three times. [^{3}H]Citalopram binding to membranes treated under the various conditions was carried out, and the K_D and Bmax determined are shown in tables 3.8 and 3.9. In addition specific [^{3}H]citalopram binding at the ligand concentration of 0.5 nM for each of the conditions are shown in figs.3.12 and 3.13.

a) Single Irradiation with 15 W (Table 3.8, Fig.3.12)

In control membranes, K_D and Bmax values for [^{3}H]citalopram binding were 0.74 ± 0.07 nM and 818.0 ± 76.2 fmol/mg protein, respectively. In the non-irradiated membranes in the presence of 5-AC, the Bmax (1064.7 ± 91.7 fmol/mg

TABLE 3.8 : Effect of Single U.V. Irradiation (15 W) on [^{3}H]Citalopram Binding

Treatment				Bmax	KD
U.V.	5-AC	UV-5-AC	N	(fmol/mg protein)	(nM)
-	-	- (control)	4	818.0 ± 76.2	0.74 ± 0.07
-	+	-	5	1064.7 ± 91.7	1.11 ± 0.12 *
-	-	+	3	811.0 ± 108.8	0.81 ± 0.15
1 min	-	-	3	908.2 ± 98.3	0.74 ± 0.10
1 min	+	-	4	896.8 ± 38.5	0.82 ± 0.07
2 min	-	-	3	853.0 ± 74.0	0.81 ± 0.08
2 min	+	-	5	782.2 ± 60.6	0.81 ± 0.04
4 min	-	-	6	710.1 ± 23.5	1.08 ± 0.22
4 min	+	-	3	675.8 ± 20.1	0.94 ± 0.17

Membranes were incubated in the absence (-) or presence (+) of 5-AC (200 nM) or UV-5-AC (200 nM) in the dark for 30 min at 25°C then irradiated with 15 W for 1, 2 or 4 min (- ; non-irradiation). After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Bmax and KD values were determined as described in table 3.5. Values are the mean ± S.E.M. from the number of separate experiments shown in table (N). * ; $P < 0.05$ compared with control.

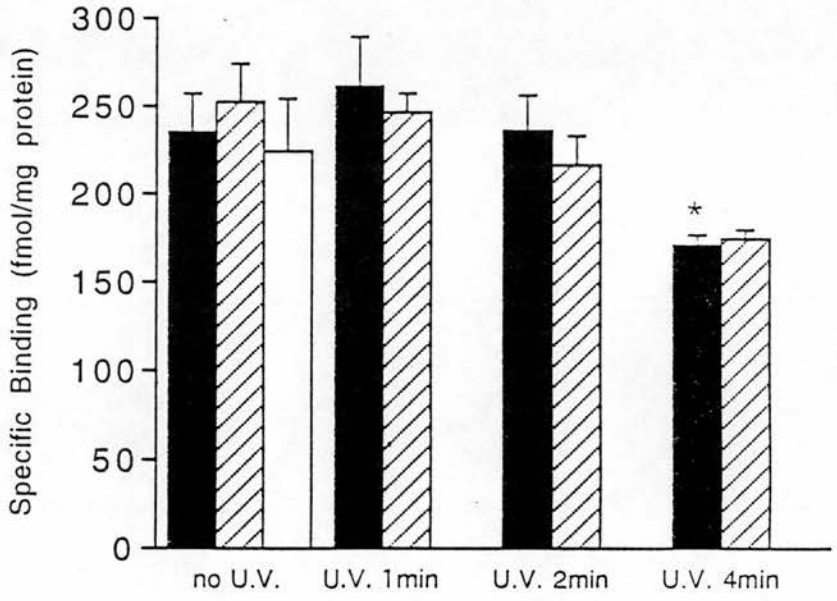


Fig. 3.12 Effect of single U.V. irradiation (15 W) on specific binding of [^{3}H]citalopram at 0.5 nM. Rat cortical membranes were incubated in the absence or presence of 5-AC (200 nM) or UV-5-AC (200 nM) in the dark for 30 min at 25°C and then irradiated with 15 W for 1, 2 or 4 min. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) for 60 min at 25°C in the absence or presence of 10 μM unlabelled citalopram. Specific binding at 0.5 nM (fmol / mg protein) was calculated from binding data. Data are the mean ± S.E.M. from 3-6 separate experiments. no U.V. : non-irradiated membranes; ■ no drug; ▨ in the presence of 5-AC; □ in the presence of UV-5-AC. * ; $P < 0.01$ compared with non-irradiated membranes in the absence of drugs.

protein) was not significantly different from that for control membranes, but the K_D value (1.11 ± 0.12 nM) was slightly and significantly higher than that for control membranes. Again these results indicate that 5-AC was not completely removed from the membranes by extensive washing and about 1 nM may have remained in the final suspension used for binding experiments. In contrast, the K_D (0.81 ± 0.15 nM) and Bmax (811.0 ± 108.8 fmol/mg protein) values for the non-irradiated membranes in the presence of UV-5-AC, were not different from those for control membranes. Thus even if small amounts similar to those remaining for 5-AC were present, due to the lower affinity of UV-5-AC very little inhibition of binding would be expected. It should also be noted that following membrane irradiation the major component being removed by washing would be UV-5-AC rather than 5-AC (Table 3.3). U.V. irradiation for 1 and 2 min in the absence of drugs did not alter Bmax (1 min : 908.2 ± 98.3 fmol/mg protein, 2 min : 853.0 ± 74.0 fmol/mg protein) when compared with that for control membranes (Table 3.8). K_D values were also similar (1 min : 0.74 ± 0.10 nM, 2 min : 0.81 ± 0.08 nM) compared with control membranes indicating that the irradiation for 1 or 2 min does not directly affect the membranes. On the other hand U.V. irradiation for 4 min in the absence of drugs produced a non significant 13% reduction of Bmax (710.1 ± 23.5 fmol/mg protein) compared with that for control membranes (Table 3.8). In addition, a significant 27% reduction ($P < 0.01$) in specific [^{3}H]citalopram binding at the ligand concentration of 0.5 nM for the 4 min irradiated membranes in the absence of drugs was observed compared with control membranes (Fig.3.12). These results indicate that the

irradiation for 4 min may directly affect the membranes. In the presence of 5-AC, irradiation (1, 2 and 4 min) did not produce a significant reduction in the Bmax for [^{3}H]citalopram binding when compared with those for other membranes (1 min : 896.8 ± 38.5 fmol/mg protein, 2 min : 782.2 ± 60.6 fmol/mg protein, 4 min : 675.8 ± 20.1 fmol/mg protein). In addition, there was no difference in K_D values between irradiated membranes in the absence and presence of 5-AC. These results suggest that 5-AC cannot produce a covalent bond at the [^{3}H]citalopram binding sites with a single irradiation with 15 W for 1 or 2 min, and 4 min may not be suitable time for irradiation.

b) Repeated Irradiation with 15 W (Table 3.9, Fig.3.13)

Under nonphotolysing conditions, there was no difference in Bmax values between membranes in the absence and presence of 5-AC or UV-5-AC (control : 675.1 ± 53.6 fmol/mg protein, 5-AC : 733.3 ± 26.9 fmol/mg protein, UV-5-AC : 675.1 ± 53.6 fmol/mg protein). However, the K_D value (0.99 ± 0.02 nM) for 5-AC treated membranes was significantly higher than those for other membranes (control : 0.52 ± 0.02 nM, UV-5-AC : 0.66 ± 0.08 nM). These results were similar to those obtained with a single 15 W irradiation. For repeated irradiation, 2 min was chosen as the irradiation time in order to avoid a direct effect on the membrane preparations as described in section 3.3.6a. In the absence of 5-AC, Bmax (665.6 ± 24.9 fmol/mg protein) and K_D (0.62 ± 0.08 nM) for irradiated membranes were not significantly different from those for control membranes, indicating that the repeated irradiation does not directly affect the membranes. In the presence of 5-AC, repeated irradiation

TABLE 3.9 : Effect of Repeated U.V. Irradiation (15 W) on [^{3}H]Citalopram Binding

Treatment					Bmax	K_D
U.V.	5-AC	UV-5-AC		N	(fmol/mg protein)	(nM)
-	-	-	(control)	3	675.1±53.6	0.52±0.02
-	+	-		3	733.3±26.9	0.99±0.02 **
-	-	+		3	687.0±55.2	0.66±0.08
2 min	-	-		3	665.6±24.9	0.62±0.08
2 min	+	-		4	531.4±25.5 *	0.70±0.06

Membranes were incubated in the absence (-) or presence (+) of 5-AC (200 nM) or UV-5-AC (200 nM) in the dark for 30 min at 25°C then irradiated with 15 W for 2 min (- ; non-irradiation). A second dose (200 nM) of 5-AC or UV-5-AC was added and the incubation and irradiation repeated. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Bmax and K_D values were determined as described in table 3.5. Values are the mean ± S.E.M. from the number of separate experiments shown in table (N). * ; $P < 0.05$, ** ; $P < 0.01$ compared with control.

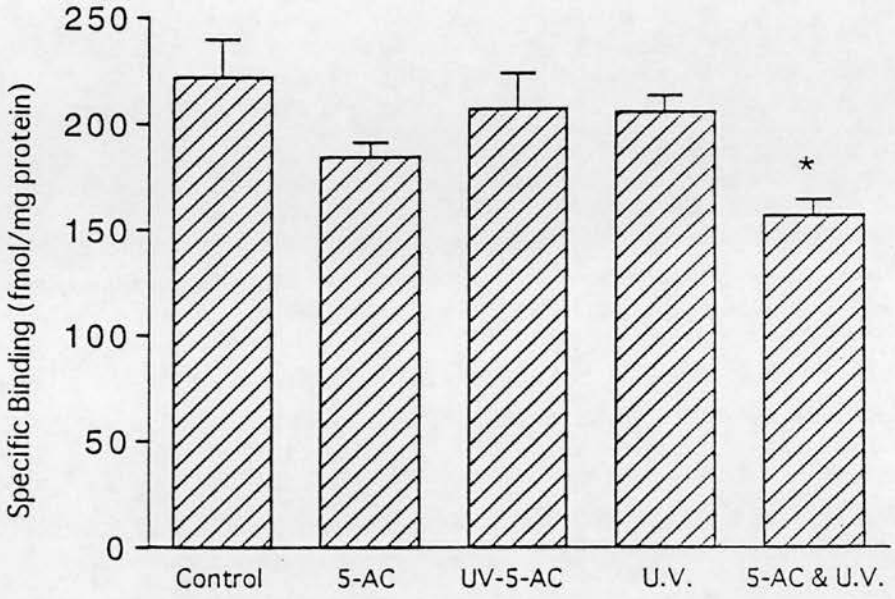


Fig. 3.13 Effect of repeated U.V. irradiation (15 W) on specific binding of [^{3}H]citalopram at 0.5 nM. Rat cortical membranes were incubated in the absence or presence of 5-AC (200 nM) or UV-5-AC (200 nM) in the dark for 30 min at 25°C and then irradiated with 15 W for 2 min. A second dose (200 nM) of 5-AC or UV-5-AC was added and the incubation and irradiation repeated. After 3 washing cycles, membranes were incubated with [^{3}H]citalopram (0.5 nM) for 60 min at 25°C in the absence or presence of 10 μM unlabelled citalopram. Specific binding at 0.5 nM (fmol / mg protein) was calculated from binding data. Data are the mean ± S.E.M. from 3 or 4 separate experiments. Control : non-irradiated membranes in the absence of drugs; 5-AC : non-irradiated membranes in the presence of 5-AC; UV-5-AC : non-irradiated membranes in the presence of UV-5-AC; U.V. : irradiated membranes in the absence of drugs; 5-AC & U.V. : irradiated membranes in the presence of 5-AC. * ; $P < 0.05$ compared with control.

showed a significant reduction (approximately 20%, $P < 0.05$ vs control) in Bmax (531.4 ± 25.5 fmol/mg protein) without alteration of K_D (0.70 ± 0.06 nM) when compared with those for control membranes or the irradiated membranes in the absence of 5-AC (Table 3.9). In addition, the specific [^{3}H]citalopram binding at 0.5 nM for the irradiated membranes in the presence of 5-AC was significantly lower than those for control membranes and the irradiated membranes in the absence of 5-AC (Fig.3.13). Thus a decrease in Bmax but no change in K_D suggests that 5-AC binds irreversibly to about 20% of the 5-HT transporter following two 2 min irradiation exposures.

3.3.8 Binding of 5-IC and [^{125}I]5-IC to the 5-HT transporters

5-IC was synthesised as a potential SPET ligand for the 5-HT transporter by Dr.I.M.Dawson, Department of Pharmacology, University of Edinburgh. The affinity of 5-IC for the 5-HT transporter was first determined *in vitro* by the potency of the unlabelled form of the compound to inhibit [^{3}H]5-HT uptake into rat cortical synaptosomes, and [^{3}H]paroxetine and [^{3}H]citalopram binding to rat cortical membranes (Table 3.10). 5-IC was 5.8-fold less potent than citalopram as an inhibitor of [^{3}H]5-HT uptake with a Ki value of 11.5 ± 1.5 nM. 5-IC inhibited binding of [^{3}H]paroxetine and [^{3}H]citalopram to rat cortical membranes with Ki values of 4.0 ± 0.9 nM and 2.9 ± 0.6 nM respectively, being 1.9- and 3.1-fold less potent than citalopram as an inhibitor of [^{3}H]paroxetine and [^{3}H]citalopram binding respectively. Thus incorporation of an iodine atom at the 5-position of citalopram produced only a small decrease in affinity for the 5-HT transporter and not sufficient to prevent

TABLE 3.10 : Effect of 5-Iodo-citalopram (5-IC) on [^{3}H]5-HT Uptake, [^{3}H]Paroxetine and [^{3}H]Citalopram Binding

	K_D/Ki (nM)		
	[^{3}H]5-HT uptake	[^{3}H]Paroxetine binding	[^{3}H]Citalopram binding
5-IC	11.5 ± 1.5 (3)	4.0 ± 0.9 (4)	2.9 ± 0.6 (4)
Citalopram	2.0 ± 0.4 (9)	2.1 ± 0.2 (9)	0.94 ± 0.24 (7)

Synaptosomes were incubated with [^{3}H]5-HT (5 nM) in the absence or presence of increasing concentrations of test drug for 5 min at 37°C. Replicate zero-time samples were carried out to define active component of [^{3}H]5-HT uptake. Membranes were incubated with [^{3}H]paroxetine (0.2 nM) or [^{3}H]citalopram (0.5 nM) in the absence or presence of increasing concentrations of test drug for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. IC_{50} values were estimated from the inhibition curves and Ki values calculated from the equation $Ki = IC_{50} / (1 + L/K_D$ or Km). Values are the mean ± S.E.M. from the number of separate experiments shown in parentheses.

further investigation of 5-IC as a potential SPET ligand.

[^{125}I]5-IC was prepared by Dr.I.M.Dawson, and experiments carried out to characterise directly binding of [^{125}I]5-IC to the 5-HT transporters. [^{125}I]5-IC (0.05 nM) was incubated with rat cortical membranes for various times at 25°C and specific binding reached equilibrium at about 60 min incubation (Fig.3.14). At equilibrium, specific binding represented about 65% of total binding. An incubation time of 60 min was therefore used for further investigation of [^{125}I]5-IC binding. The concentration dependence of [^{125}I]5-IC was investigated by incubating 0.05 nM in the presence of increasing concentrations of unlabelled 5-IC (Fig.3.15). Binding was saturable and the Hill co-efficient was close to unity (Table 3.11a) indicating a single population of sites. Analysis of data from three separate experiments gave a K_D value of 1.87 ± 0.25 nM and a Bmax value of 0.51 ± 0.12 pmol/mg protein. The Bmax value for [^{125}I]5-IC binding was not significantly different from those for [^{3}H]citalopram binding (P = 0.157) and [^{3}H]paroxetine binding (P = 0.064), suggesting that it was labelling the same population of binding sites. The pharmacology of [^{125}I]5-IC is shown in table 3.11b. The selective 5-HT uptake inhibitors, paroxetine and citalopram had high affinity for [^{125}I]5-IC binding sites with Ki values of 0.12 ± 0.01 nM and 1.42 ± 0.18 nM respectively, whereas 5-HT (Ki = 248 ± 47 nM) was a relatively weak inhibitor of [^{125}I]5-IC binding. The Ki/K_D values for paroxetine, citalopram, 5-IC and 5-HT were consistent with results obtained from binding experiments using [^{3}H]paroxetine, [^{3}H]citalopram and [^{125}I]5-IC as ligands thus confirming that the [^{125}I]5-IC binding site is the 5-HT transporter (Table 3.11b).

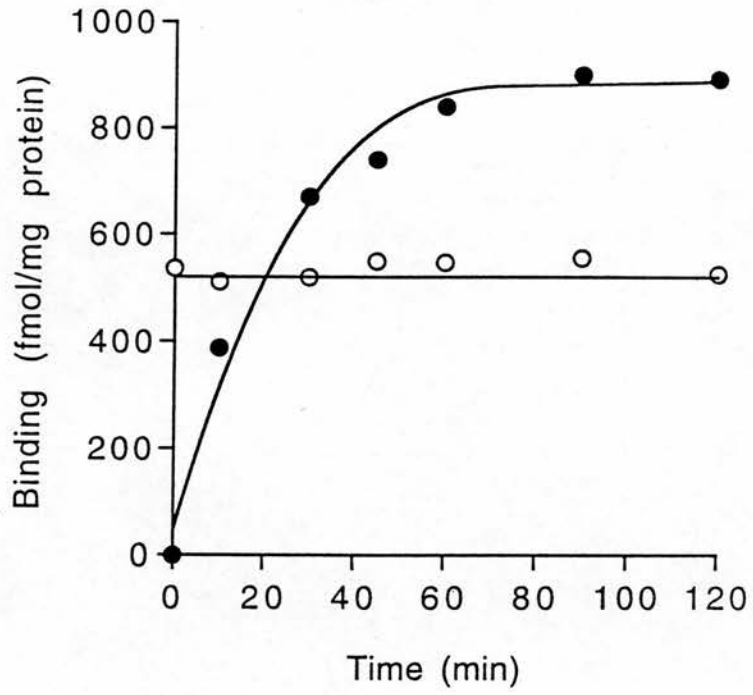


Fig. 3.14 Time course of 0.05 nM [^{125}I]5-IC binding to rat cortical membranes. Membranes were incubated with [^{125}I]5-IC for various times at 25°C. Non-specific binding was determined in the presence of 10 µM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations. ● Specific binding; ○ Non-specific binding

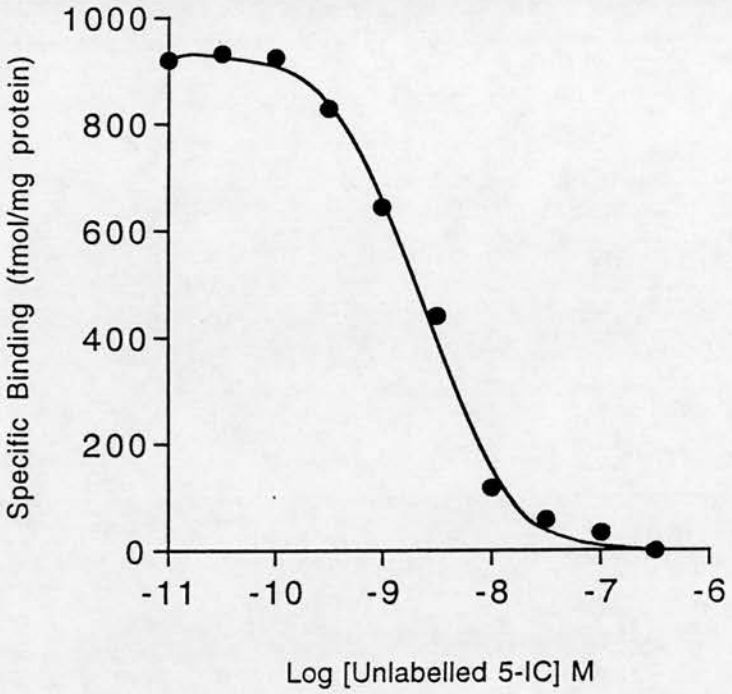


Fig. 3.15 Inhibition of [^{125}I]5-IC binding to rat cortical membranes by 5-IC. Membranes were incubated with [^{125}I]5-IC (0.05 nM) in the absence or presence of increasing concentrations of 5-IC (10^{-11} M to $3x10^{-7}$ M, final concentrations) for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Data are from a representive experiment. Each point is the mean value of duplicate determinations.

TABLE 3.11 : a) K_D / Bmax / nH values and b) Inhibition of [^{125}I]5-IC Binding to Rat Cortical Membranes

a)

	[^{125}I]5-IC binding	[^{3}H]Citalopram binding	[^{3}H]Paroxetine binding
K_D (nM)	1.87 ± 0.25 (3)	0.94 ± 0.24 (7)	0.20 ± 0.02 (6)
Bmax (pmol/mg protein)	0.51 ± 0.12 (3)	0.85 ± 0.13 (7)	0.82 ± 0.08 (6)
nH	0.98 ± 0.11 (3)	0.95 ± 0.11 (7)	1.10 ± 0.13 (6)

b)

	Ki (nM)		
	[^{125}I]5-IC binding	[^{3}H]Citalopram binding	[^{3}H]Paroxetine binding
5-HT	248 ± 47 (3)	349 ± 35 (4)	836 ± 72 (4)
Citalopram	1.42 ± 0.18 (3)	0.94 ± 0.24 (7)	2.10 ± 0.20 (9)
Paroxetine	0.12 ± 0.01 (3)	0.12 ± 0.01 (7)	0.20 ± 0.02 (6)
5-IC	1.87 ± 0.25 (3)	2.90 ± 0.60 (4)	4.00 ± 0.90 (4)

Membranes were incubated with [^{125}I]5-IC (0.05 nM), [^{3}H]citalopram (0.5 nM) and [^{3}H]paroxetine (0.2 nM) in the absence or presence of increasing concentrations of test drug for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. IC_{50} and nH (P) values were obtained from analysis of the inhibition curves from the equation $Y = MX^P / (X^P + IC_{50})$ and K_D values calculated ($K_D = IC_{50}$ - [^{3}H]ligand). Bmax values were determined from the equation $b = Bmax\ L / (L + K_D)$. Ki values calculated from the equation $Ki = IC_{50} / (1 + L/K_D)$. Values are the mean ± S.E.M. from the number of separate experiments shown in parentheses.

3.3.9 In Vivo Distribution of [^{125}I]5-IC

The time course of the distribution of [^{125}I]5-IC following intravenous injection into the femoral vein of rats was examined using a tracer dose of 20 μCi [^{125}I]5-IC corresponding to 20 nmoles. The blood level of radioactivity of [^{125}I]5-IC is shown in table 3.12 and fig.3.16. After intravenous administration, the arterial blood level was 2.351 %D/ml at 1 min, followed by a biphasic decline with half lives of 0.68 min (α) and 137.50 min (β) (Table 3.15). The AUC for blood was 42.185 %D · min/ml. The tissue level of radioactivity at various time points after [^{125}I]5-IC injection is shown in tables 3.13, 3.14 and figs.3.17, 3.18, 3.19. In kidney, lung and thyroid, peak levels were obtained by 30 min, whereas the peak levels in heart and liver were observed at 60 min (Table 3.13, Fig.3.17). At 30 and 60 min after [^{125}I]5-IC injection, these peripheral tissues contained radioactivity concentrations greater than that of blood. Among peripheral tissues, liver, lung and kidney had higher concentrations of radioactivity compared with thyroid and heart as indicated by their AUC values (liver : 4883.719, lung : 1068.152, kidney : 591.453, heart : 161.300, thyroid : 156.494 %D · min/g) (Table 3.15). The clearance of radioactivity from peripheral tissues was relatively slow with a $t_{1/2}$ value of 286.36 min (heart), 161.92 min (kidney), 1237.5 min (liver), 202.63 min (lung) and 190.38 min (thyroid) (Table 3.15).

Uptake of [^{125}I]5-IC into whole brain reached a maximum between 30 min and 1 hr at approximately 0.2 %D/g (Table 3.14, Fig.3.18). Even at 5 hr after injection, significant amounts of radioactivity were still observed at approximately 0.04 %D/g. In all six brain regions, the amount of radioactivity was highest

TABLE 3.12 : Distribution of [^{125}I]5-IC in Blood

	% injected dose / ml time (min)											
	1	2	3	5	10	20	30	45	60	90	180	300
a)	2.351 (2)	0.581 (2)	0.269 (2)	0.215 (2)	0.183 (2)	0.166 (2)	0.153±0.012 (4)	0.146 (2)	0.152±0.011 (4)	0.172 (2)	0.214 (2)	0.192 (2)
b)	-	-	-	-	-	-	0.114 (2)	-	0.134 (2)	0.134 (2)	0.092 (2)	0.177 (2)

Rats were injected with 20 μCi (20 nmol) [^{125}I]5-IC via the right/left femoral vein, and the blood was collected at various time points from a) femoral artery or b) inferior vena cava. The radioactivity of blood (100 μl) was determined using gamma spectrometry. Data are the mean value ± S.E.M. of 4 animals or the mean value of 2 animals. The number of animals is shown in parentheses.

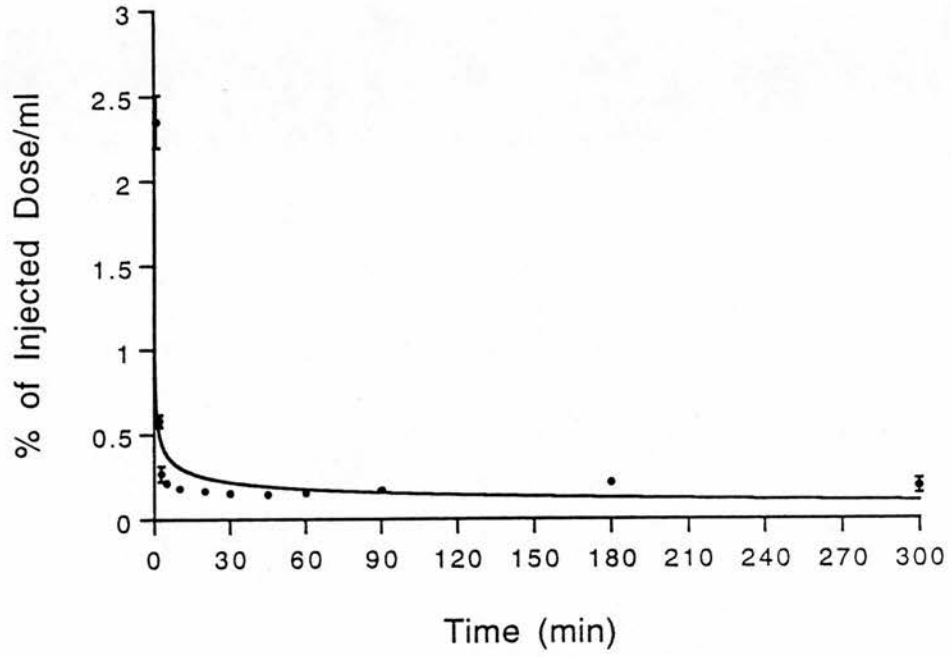


Fig. 3.16 Time course of radioactivity in arterial blood after [^{125}I]5-IC injection. Under anaesthesia, rats were injected with 250 µl of saline containing 20 µCi (20 nmol) of [^{125}I]5-IC, 10% ethanol and 1% Tween-80 through a cannula placed in the left femoral vein. The arterial blood (300 µl) was collected at various time points (1, 2, 3, 5, 10, 20, 30, 45, 60, 90, 180 and 300 min after injection) via the femoral artery and the radioactivity in 100 µl of blood was determined using a Packard Cobra auto-gamma counter. Data are expressed as % injected dose per ml. Each time point is the mean value ± S.E.M. of 4 animals for the mean value of 2 animals. The number of animals is shown in table 3.12

TABLE 3.13 : Distribution of [^{125}I]5-IC in Peripheral Tissues

Tissue	% injected dose / g tissue time (min)				
	30	60	90	180	300
Heart	0.397, 0.319 (0.356)	0.448, 0.329 (0.388)	0.221, 0.318 (0.269)	0.255, 0.196 (0.226)	0.201, 0.194 (0.197)
Kidney	2.742,1.802 (2.272)	2.416,1.724 (2.070)	1.830,1.198 (1.514)	1.421,1.018 (1.220)	0.811,0.580 (0.696)
Liver	1.854, 2.285 (2.070)	2.910, 2.348 (2.629)	2.665, 2.606 (2.635)	2.681, 2.230 (2.455)	2.588, 2.051 (2.320)
Lung	4.272, 3.560 (3.916)	3.602, 2.444 (3.023)	2.001, 2.135 (2.068)	1.808, 1.632 (1.720)	1.274, 1.576 (1.425)
Thyroid	0.496, 0.436 (0.466)	0.443, 0.451 (0.447)	0.437, 0.450 (0.444)	0.332, 0.328 (0.330)	0.188, 0.165 (0.177)

Rats were injected with 20 μCi (20 nmol) [^{125}I]5-IC via femoral vein and killed at various time points. Organs were removed and weighed. The whole thyroid was taken for counting. Samples of other organs were taken (100-250 mg), weighed and counted. Data are expressed as % injected dose / g tissue, and the mean values are shown in parentheses.

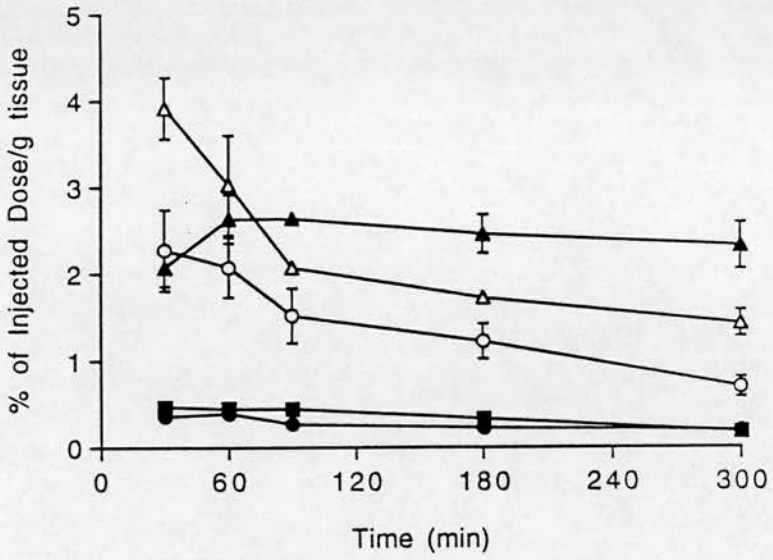


Fig. 3.17 Time course of radioactivity in peripheral tissues after [^{125}I]5-IC injection. Under anaesthesia, rats were injected with 250 µl of saline containing 20 µCi (20 nmol) of [^{125}I]5-IC, 10% ethanol and 1% Tween-80 through a cannula placed in the left femoral vein. At various times post-injection (30, 60, 90, 180 and 300 min), rats were killed, and liver, kidney, lung, thyroid and heart were removed and weighed. The radioactivity in the thyroid and 100-200 mg weighed samples of the other tissues were determined using a Packard Cobra auto-gamma counter. Data are expressed as % injected dose per g tissue. Each time point is the mean value ± range from 2 animals. ● Heart; ○ Kidney; ▲ Liver; △ Lung; ■ Thyroid.

TABLE 3.14 : Distribution of [^{125}I]5-IC in Brain

a) % injected dose / g tissue

b) amol / mg tissue

Brain region	time (min)					
	30	60	90	180	210	300
Whole brain	a) 0.254, 0.183 (0.218)	0.269, 0.157 (0.213)	0.153,0.127 (0.140)	0.077,0.059 (0.068)		0.044,0.037 (0.040)
	b) 19.025,13.688 (16.356)	20.129,11.782 (15.955)	11.439,9.538 (10.489)	5.755,4.427 (5.091)		3.274,2.760 (3.017)
Rostral cortex	a) 0.270, 0.333 (0.320)	0.336, 0.270 (0.272)	0.182,0.155 (0.168)	0.093,0.084 (0.087)	0.096	
	b) 17.303,21.362 (19.333)	21.544,13.301 (17.423)	11.650,9.904 (10.777)	5.982,5.364 (5.673)	6.165	
Caudal cortex	a) 0.279, 0.334 (0.307)	0.303, 0.199 (0.251)	0.203,0.145 (0.174)	0.089,0.077 (0.083)	0.099	
	b) 17.911,21.395 (19.653)	19.451,12.727 (16.089)	12.998,9.283 (11.141)	5.687,4.949 (5.318)	6.329	
Hippocampus	a) 0.243, 0.347 (0.295)	0.310, 0.198 (0.254)	0.200,0.153 (0.177)	0.108,0.091 (0.100)	0.114	
	b) 15.606,22.218 (18.912)	19.908,12.678 (16.293)	12.853,9.794 (11.324)	6.927,5.835 (6.381)	7.330	
Brainstem	a) 0.242, 0.284 (0.263)	0.329, 0.206 (0.267)	0.190,0.097 (0.143)	0.098,0.084 (0.091)	0.102	
	b) 15.512,18.228 (16.870)	21.070,13.224 (17.147)	12.174,6.211 (9.193)	6.311,5.364 (5.838)	6.561	
Midbrain	a) 0.270, 0.345 (0.307)	0.317, 0.249 (0.283)	0.180,0.150 (0.165)	0.093,0.080 (0.087)	0.094	
	b) 17.289,22.117 (19.703)	20.317,15.996 (18.157)	11.518,9.597 (10.558)	5.955,5.142 (5.549)	6.056	
Cerebellum	a) 0.251, 0.315 (0.283)	0.277, 0.156 (0.217)	0.128,0.112 (0.120)	0.060,0.061 (0.061)	0.073	
	b) 16.116,20.226 (18.171)	17.790,10.018 (13.904)	8.174,7.211 (7.693)	3.836,3.921 (3.879)	4.677	

Rats were injected with 20 μCi (20 nmol) [^{125}I]5-IC via femoral vein and killed at various time points.
Data are expressed as a)% injected dose / g tissue and b) amol / mg tissue, and the mean values are shown in parentheses.

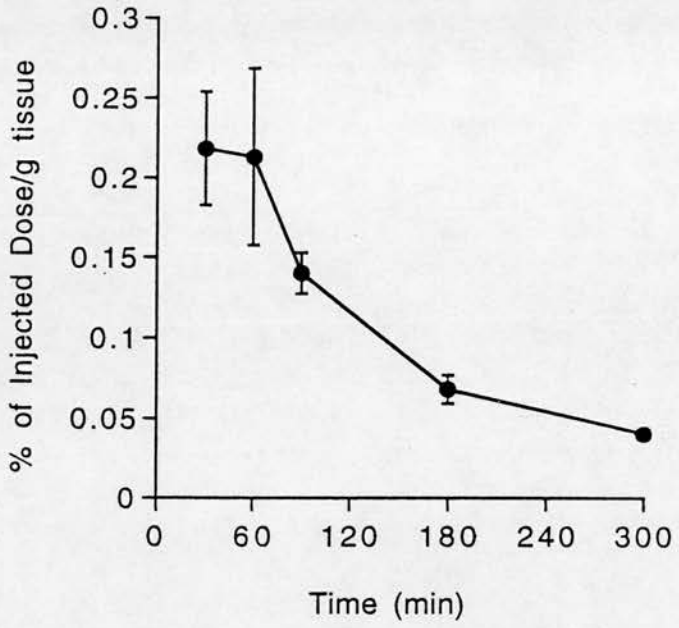


Fig. 3.18 Time course of radioactivity in whole brain after [^{125}I]5-IC injection. Under anaesthesia, rats were injected with 250 µl of saline containing 20 µCi (20 nmol) of [^{125}I]5-IC, 10% ethanol and 1% Tween-80 through a cannula placed in the left femoral vein. At various times post-injection (30, 60, 90, 180 and 300 min), rats were killed, and the brains removed. The radioactivity was determined in a Packard Cobra auto-gamma counter. Data are expressed as % injected dose per g tissue. Each time point is the mean value ± and range for 2 animals.

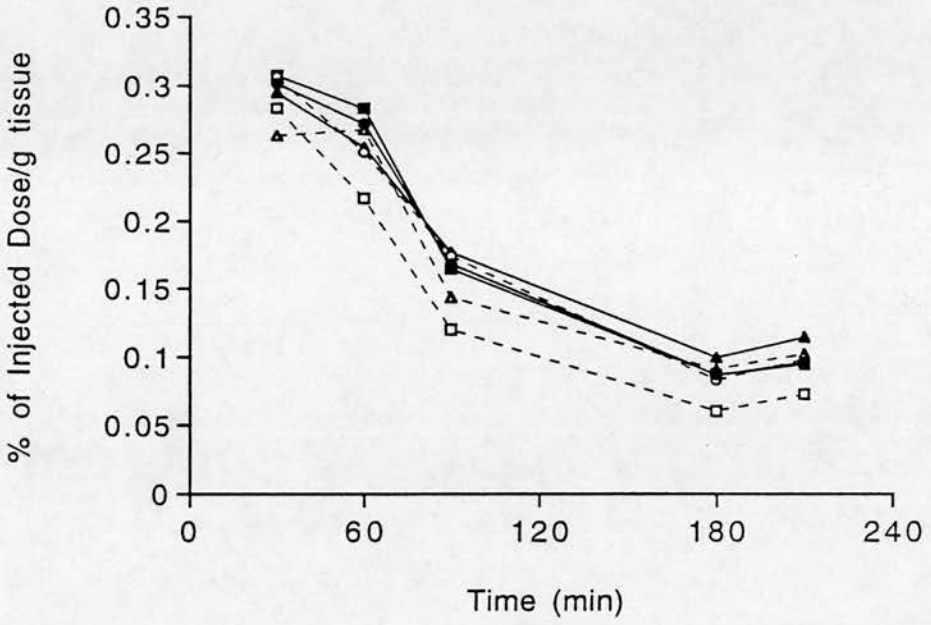


Fig. 3.19 Time course of radioactivity in brain regions after [^{125}I]5-IC injection. Under anaesthesia, rats were injected with 250 µl of saline containing 20 µCi (20 nmol) of [^{125}I]5-IC, 10% ethanol and 1% Tween-80 through a cannula placed in the left femoral vein. At various times post-injection (30, 60, 90, 180 and 210 min), rats were killed, and the brains removed, divided into 6 regions : rostral cortex, caudal cortex (the remainder of the cortex), hippocampus, brainstem, midbrain and cerebellum. These brain areas were weighed and radioactivity determined in a Packard Cobra auto-gamma counter. Data are expressed as % injected dose per g tissue. Each time point is the mean value of 2 animals. ● Rostral Cortex; ○ Caudal Cortex; ▲ Hippocampus; △ Brainstem; ■ Midbrain; □ Cerebellum.

TABLE 3.15 : Pharmacokinetic Parameters of [^{125}I]5-IC in Rats after Injection

	Co (% D/g or ml)	Ke (min^{-1})	$t_{1/2}$ (min)	AUC (% D · min/g or ml)
Whole brain	0.271	0.00669	103.59	40.910
Rostral cortex	0.368	0.00715	96.92	52.908
Caudal cortex	0.362	0.00707	98.02	53.082
Hippocampus	0.337	0.00590	117.46	59.174
Brainstem	0.313	0.00610	113.61	52.882
Midbrain	0.379	0.00736	94.16	52.685
Cerebellum	0.321	0.00814	85.14	40.734
Heart	0.382	0.00242	286.36	161.300
Kidney	2.521	0.00428	161.92	591.453
Liver	2.737	0.00056	1237.5	4883.719
Lung	3.563	0.00342	202.63	1068.152
Thyroid	0.569	0.00364	190.38	156.494
Blood	3.658	1.02	0.68	42.185
	0.186	0.00504	137.50	

The data of radioactivity in blood after [^{125}I]5-IC injection were fitted to biexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-\alpha t)} + B \times e^{(-\beta t)}$, where t is time after administration, Y is the measured radioactivity, A and B are the coefficients of the exponential terms, α is the apparent distribution rate constant, β is the apparent terminal rate constant. The data of radioactivity in brain and peripheral tissues were fitted to monoexponential functions using least squares regression analysis. The equation used was $Y = A \times e^{(-Ke \cdot t)}$, where Ke is the elimination rate constant. Co : radioactivity at t = 0, $t_{1/2}$ (half-life) = 0.693/Ke or α or β, AUC : the area under the radioactivity concentration-time curve from zero to time infinity.

at 30 or 60 min (Table 3.14, Fig.3.19) and all brain regions had similar radioactivity elimination half-lives (rostral cortex : 96.92 min, caudal cortex : 98.02 min, hippocampus : 117.46 min, brainstem : 113.61 min, midbrain : 94.16 min, cerebellum : 85.14 min) (Table 3.15). At 30 min, the radioactivity concentration was similar in all brain regions but at all other time points the radioactivity concentrations in the cerebellum was lower in comparison with other 5 brain regions (Table 3.14, Fig.3.19). The AUC value (40.734 %D · min/g) for cerebellum was smallest among the 6 brain regions (rostral cortex : 52.908, caudal cortex : 53.082, hippocampus : 59.174, brainstem : 52.882, midbrain : 52.685 %D · min/g) (Table 3.15). At 2 hr after injection, the radioactivity for cerebellum (0.0732 %D/g) was significantly lower than those for other five brain regions (Table 3.16). The ratios of the brain regions to cerebellum were 1.37 (rostral cortex), 1.32 (caudal cortex), 1.46 (hippocampus), 1.58 (brainstem) and 1.51 (midbrain). In order to determine the proportion of the [^{125}I]5-IC found in each brain which was bound in vivo to the 5-HT transporters, animals were pretreated with paroxetine (1 mg/kg, i.v.) and the distribution of [^{125}I]5-IC in the various brain regions measured at 2 hr. Compared with control tissue, pretreatment of paroxetine reduced the radioactivity in rostral cortex, caudal cortex, hippocampus, brainstem and midbrain, whereas it did not reduce the radioactivity in cerebellum (Table 3.16). Therefore it was considered that the radioactivity in the cerebellum represented apparent nonspecific binding and apparent specific binding was calculated by subtracting the radioactivity in the cerebellum from that in other brain regions (Table 3.16). The highest level of specific

TABLE 3.16 : Effect of Paroxetine on In Vivo [^{125}I]5-IC Binding in Rat Brain

Brain region	% injected dose / g tissue			
	Control	Paroxetine	Control (Brain region - Cerebellum)	Paroxetine (Brain region - Cerebellum)
Rostral cortex	0.1002 ± 0.0028 ** (1.37)	0.0891 ± 0.0077 (1.07)	a) 0.0220 ± 0.0048 b) 0.0270 ± 0.0005	0.0109 ± 0.0133 0.0059 ± 0.0032 ##
Caudal cortex	0.0963 ± 0.0033 ** (1.32)	0.0917 ± 0.0080 (1.10)	a) 0.0181 ± 0.0057 b) 0.0231 ± 0.0018	0.0135 ± 0.0139 0.0085 ± 0.0035 #
Hippocampus	0.1070 ± 0.0032 ** (1.46)	0.1054 ± 0.0079 (1.27)	a) 0.0288 ± 0.0047 b) 0.0339 ± 0.0001	0.0272 ± 0.0131 0.0221 ± 0.0045 ##
Brainstem	0.1153 ± 0.0068 ** (1.58)	0.1044 ± 0.0057 (1.25)	a) 0.0371 ± 0.0116 b) 0.0422 ± 0.0070	0.0262 ± 0.0099 0.0212 ± 0.0014 #
Midbrain	0.1104 ± 0.0064 ** (1.51)	0.0890 ± 0.0060 (1.07)	a) 0.0322 ± 0.0111 b) 0.0372 ± 0.0066	0.0108 ± 0.0104 0.0058 ± 0.0027 #
Cerebellum	0.0732 ± 0.0029	0.0832 ± 0.0062	-------	-------

Rats were injected with either saline (control) or paroxetine (1 mg/kg) via a femoral vein 5 min prior to injection of 20 μCi [^{125}I]5-IC, and killed at 2 hr post-injection. Values (% injected dose / g tissue) are the mean ± S.E.M. from 3 animals. The ratios (brain region / cerebellum) are shown in parentheses. ** ; $P < 0.01$ compared with cerebellum, # ; $P < 0.05$, ## ; $P < 0.01$ compared with Control (Brain region - Cerebellum). (Brain region - Cerebellum) are calculated by a) individual brain region - average of control and paroxetine treated cerebellum (0.0782 %D/g, n = 6) or b) individual brain region - individual cerebellum.

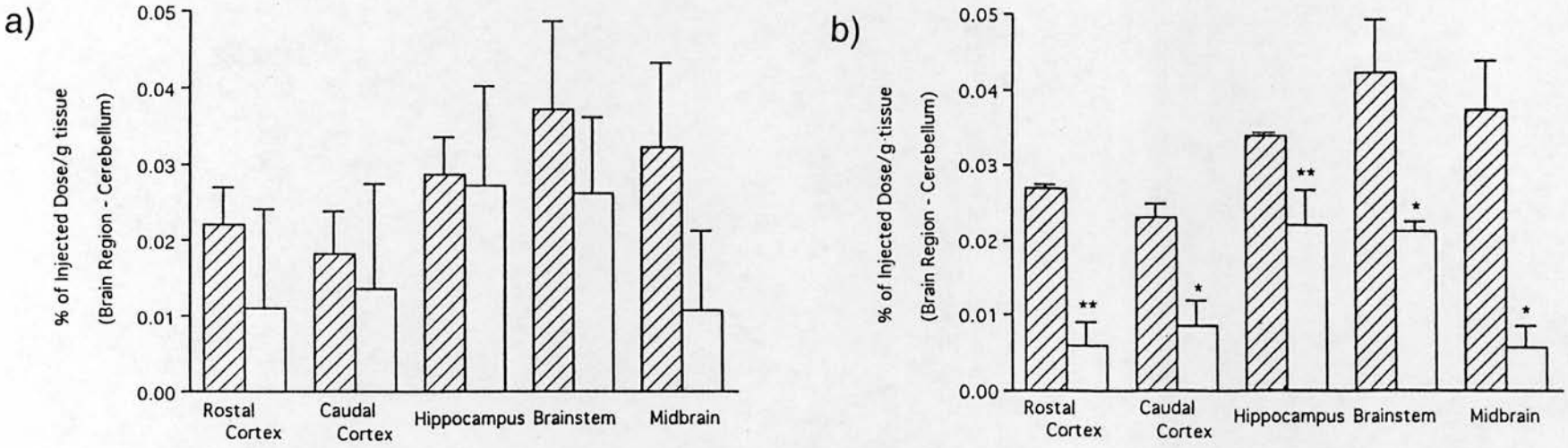


Fig. 3.20 Effect of paroxetine on <u>in vivo</u> [^{125}I]5-IC binding in different brain regions. Under anaesthesia, rats were injected with either saline (control) or paroxetine (1 mg/kg) through a cannula placed in the left femoral vein 5 min prior to injection of 250 µl of saline containing 20 µCi (20 nmol) of [^{125}I]5-IC, 10% ethanol and 1% Tween-80, and killed at 2 hr later. The brains were removed, divided into 6 regions : rostral cortex, caudal cortex (the remainder of the cortex), hippocampus, brainstem, midbrain and cerebellum. The radioactivity in each brain region was determined as described in fig.3.19. The radioactivity in the cerebellum was designated as apparent nonspecific binding because it was not reduced by pretreatment with paroxetine (Table 3.16). Therefore apparent specific binding was calculated by subtracting the radioactivity in the cerebellum from the radioactivity in each brain region. (Brain region - Cerebellum) are calculated by a) individual brain region - average of control and paroxetine treated cerebellum (0.0782 %D/g, n = 6) or b) individual brain region - individual cerebellum. Values (% injected dose/g tissue) are the mean ± S.E.M. from 3 animals. ▨ Control; □ Paroxetine. * ; $P < 0.05$, ** ; $P < 0.01$ compared with control.

binding was observed in the brainstem (0.042 %D/g) and midbrain (0.037 %D/g), areas which are known to be rich in serotonergic neurons. Pretreatment with paroxetine caused a significant reduction in specific [^{125}I]5-IC binding in all brain regions 2hr postinjection (Table 3.16, Fig.3.20). The greatest reductions were observed in the midbrain (84.4%), rostral cortex (78.1%) and caudal cortex (63.2%). Smaller reductions occurred in the brainstem (49.8%) and hippocampus (34.8%). Thus a brain regional variation in the inhibitory effect of paroxetine was observed.

3.4 DISCUSSION

In order to evaluate appropriate compounds in vitro as photoaffinity and as SPET ligands for the 5-HT transporter, assay for [^{3}H]5-HT uptake into rat cortical synaptosomes and the binding of [^{3}H]paroxetine and [^{3}H]citalopram to rat cortical membranes were established. For [^{3}H]5-HT uptake into rat cortical synaptosomes, potent inhibition of [^{3}H]5-HT uptake was exhibited only by selective 5-HT uptake inhibitors such as paroxetine (Ki = 0.83 nM), citalopram (Ki = 2.0 nM) and fluoxetine (Ki = 11.3 nM). Binding of [^{3}H]paroxetine to rat cortical membranes was also inhibited by low concentrations of the selective 5-HT uptake inhibitors (paroxetine : K_D = 0.20 nM, citalopram : Ki = 2.1 nM, fluoxetine : Ki = 6.1 nM). As with [^{3}H]5-HT uptake and [^{3}H]paroxetine binding, paroxetine (Ki = 0.12 nM), citalopram (K_D = 0.94 nM) and fluoxetine (Ki = 6.3 nM) were potent inhibitors of [^{3}H]citalopram binding to rat cortical membranes. The similar Bmax values for [^{3}H]paroxetine (0.82 pmol/mg protein) and [^{3}H]citalopram binding (0.85 pmol/mg

protein) indicate that they probably label the same population of sites. In addition, there was a good correlation between the potency of various drugs to inhibit [^{3}H]5-HT uptake, [^{3}H]paroxetine and [^{3}H]citalopram binding (Fig.3.21). The relative potencies of drugs in inhibiting [^{3}H]paroxetine binding closely parallel their inhibitory effect on [^{3}H]citalopram binding with a correlation coefficient (r) of 0.995 and a slope of 1.05 ($p < 0.05$). On the other hand, the correlations between results for [^{3}H]5-HT uptake and [^{3}H]paroxetine or [^{3}H]citalopram binding were not significant ($r = 0.869$ ($P = 0.153$), $r = 0.898$ ($P = 0.110$) respectively). However, their correlations for 5 drugs but excluding 5-HT were excellent with r values of 0.970 (slope = 0.74, $P < 0.05$, [^{3}H]paroxetine binding vs [^{3}H]5-HT uptake) and 0.985 (slope = 0.76, $P < 0.05$, [^{3}H]citalopram binding vs [^{3}H]5-HT uptake). Thus a difference in affinity for 5-HT, the only substrate for the carrier tested, between 5-HT uptake and ligand binding assays was observed. This may reflect the difference in measurement of an equilibrium dissociation constant in the ligand binding assay compared with a Michaelis constant for [^{3}H]5-HT uptake into serotonergic neurones which may involve more than one step. The binding of 5-HT to the surface of the 5-HT transporter is only the first step of the uptake process and therefore the K_D value and Km value (a steady state constant) may not reflect identical physiological processes involved in the uptake of 5-HT (Marcusson et al., 1986). It is concluded that all three assays were established and can be used for measurement of drug interaction at the amine transporter in serotonergic neurones.

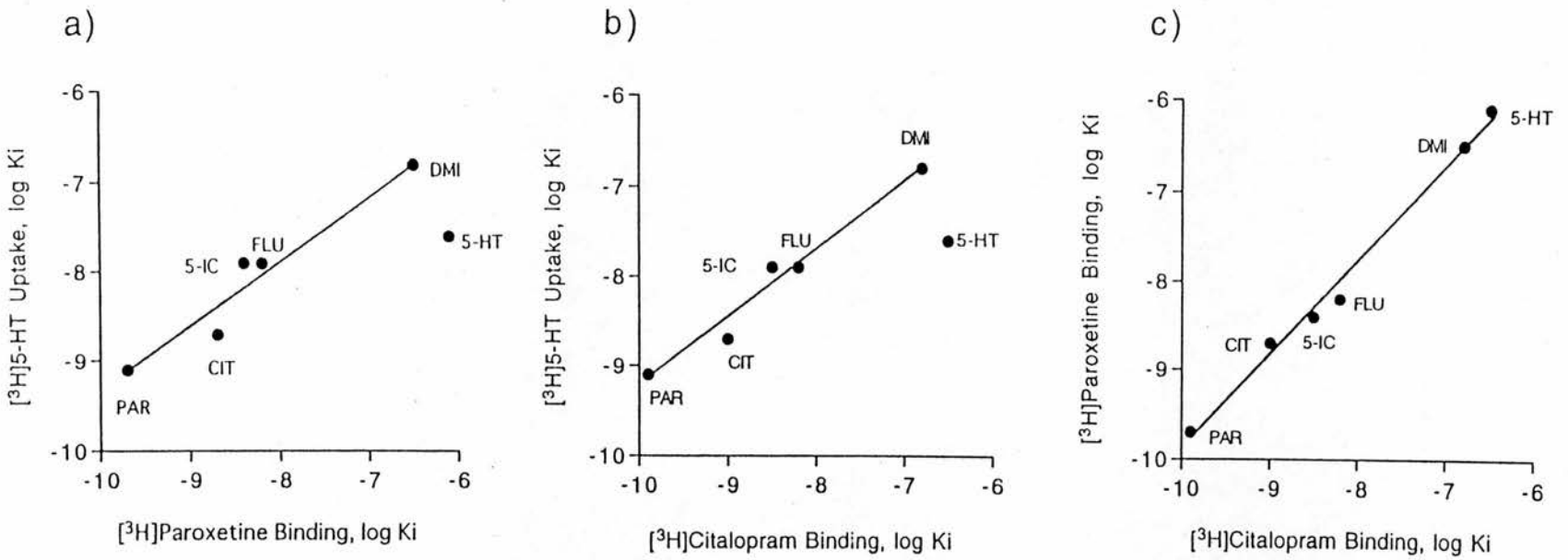


Fig. 3.21 Correlation between a) potency for inhibition of [3H]paroxetine binding and potency for inhibition of [3H]5-HT uptake, b) potency for inhibition of [3H]citalopram binding and potency for inhibition of [3H]5-HT uptake, c) potency for inhibition of [3H]citalopram binding and potency for inhibition of [3H]paroxetine binding. The Ki values for inhibition of [3H]5-HT uptake into rat cortical synaptosomes and of [3H]paroxetine and [3H]citalopram binding to rat cortical membranes by the various drugs are taken from tables 3.2 and 3.10. The correlation coefficient (r) was 0.970 for a), 0.985 for b) and 0.995 for c). The slope value was 0.74 for a), 0.76 for b) and 1.05 for c). The correlation coefficients and slope values for a) and b) were determined from data excluding 5-HT. PAR : paroxetine, CIT : citalopram, FLU : fluoxetine, DMI : desipramine, 5-IC : 5-iodo-citalopram.

The effect of 5-azido-citalopram (5-AC) synthesised as a candidate for a photoaffinity ligand was examined using [^{3}H]citalopram binding to rat cortical membranes. 5-AC had high affinity (Ki = 1.65 nM) for [^{3}H]citalopram binding which was only 1.8-fold less potent than citalopram itself. Thus the azide moiety can be incorporated at the 5 position of citalopram almost without loss of biological activity compared with citalopram.

In order to investigate the potential use of 5-AC as a photoaffinity ligand, the ability of photolysis to induce 5-AC to covalently bind to the 5-HT transporter was examined. Initially, the most suitable U.V. irradiation time was determined using 5-AC in assay buffer in the absence of membranes. Initially, a U.V. lamp (400 W) with a 365 nm wavelength was used to irradiate solutions of 5-AC. One minute irradiation decomposed 5-AC completely whereas 50% of 5-AC was decomposed by irradiation for 30 sec. Thus 5-AC was decomposed rapidly and time-dependently by U.V. irradiation with 400W.

To examine whether 5-AC bound irreversibly to the 5-HT transporter following 1 min U.V. irradiation, binding of [^{3}H]citalopram was compared using rat cortical membranes treated under various conditions. In the presence of 5-AC (200 nM), 1 min irradiation showed no reduction in Bmax for [^{3}H]citalopram binding to rat cortical membranes compared with those for control membranes, non-irradiated membranes in the presence of 5-AC or irradiated membranes in the absence of 5-AC. These results indicate that 5-AC did not bind irreversibly to the 5-HT transporter following photolysis for 1 min.

Since it is known that repeated U.V. irradiation is often more effective than single irradiation (Bayley and Knowles, 1977; Gozlan et al., 1987), the effect of repeated U.V. irradiation (30 sec x 2) on [^{3}H]citalopram binding was examined. In the presence of 5-AC, repeated irradiation produced a significant reduction in specific [^{3}H]citalopram binding to rat cortical membranes. When compared with control membranes or irradiated membranes in the absence of 5-AC, the reduction was 73.6% and 74.3%, respectively. These results suggest that a covalent bond between 5-AC and the [^{3}H]citalopram binding sites was formed by repeated U.V. irradiation. However, there was a variation in the percentage reduction of specific [^{3}H]citalopram binding between separate experiments (data not shown). In some experiments, 100% reduction was observed but only a 54% reduction was observed in other experiments. These variations between experiments may be partly due to the amount of 5-AC remaining in the membranes either to differences in the efficiency of the extensive washing cycles or, because irradiation for 30 sec does not completely decompose 5-AC, to differences in the proportion of 5-AC remaining after irradiation (Table 3.3). A greater proportion of 5-AC remaining in the membrane would have produced more inhibition of [^{3}H]citalopram binding since it has 9-fold higher affinity than UV-5-AC for the transporter. Substantial contamination of the final membrane preparation with 5-AC and/or UV-5-AC would have been reflected in a higher apparent K_D value for [^{3}H]citalopram binding compared with control. Paradoxically due to the low amounts of [^{3}H]citalopram binding in irradiated membranes in the presence of 5-AC, it was not possible to

accurately determine K_D and Bmax values. However it can be calculated that for 100% inhibition of [^{3}H]citalopram binding by 5-AC or UV-5-AC at least 10-fold of their Ki concentrations would have been needed to be present (16.5 nM and 142.5 nM respectively). This is considered unlikely since the washing procedure following incubation with 200 nM 5-AC was estimated to reduce the concentration to 1 nM or below (Table 3.6). It should also be noted that following membranes being incubated with 200 nM UV-5-AC then washing, no effect on K_D and Bmax values for [^{3}H]citalopram binding was observed (Table 3.9). In conclusion although it was not possible to obtain stable and reproducible results using photolysis with 400 W at least a proportion of the transporters up to 70% may have been covalently bound with 5-AC.

In order to confirm whether 5-AC can form a covalent bond with the 5-HT transporter induced by irradiation, an other type of U.V. lamp (15 W, 254 nm) was used for photolysis. This wavelength (254 nm) is known to be suitable for photolysis of aryl azides (Bayley and Knowles, 1977). Studies of decomposition of aryl azides, without membranes present, by photolysis with a 254 nm source have been reported by some investigators (Hess et al., 1983; Niznik et al., 1984; Hawkinson et al., 1991). An azide analogue of the α_1-adrenergic receptor antagonist prazocin, CP59430 is decomposed completely using 5 min irradiation (Hess et al., 1983). The photoaffinity ligand (N_3TBOB) for GABA-A receptors has been shown to decompose using 3 min photolysis (Hawkinson et al., 1991). In addition Niznik et al. (1984) showed that azidoclebopride used as a photoaffinity ligand for dopamine D_2 receptors is almost

completely decomposed after irradiation for 30 sec. The differences in irradiation time for decomposition between these reports might be due to the chemical structures or to experimental conditions such as intensity of the light source, distance from light source and concentration of compound. In the present experiments, irradiation (15 W, 254 nm) for 2 min decomposed 5-AC completely whereas 98% and 81% of 5-AC was decomposed by irradiation for 1 min and 30 sec, respectively. Compared with 400 W (365 nm), 15 W (254 nm) was more effective for decomposition of 5-AC (Table 3.3), indicating that 254 nm may be suitable for photolysis of aryl azides as described above.

Using the 15 W U.V. lamp, the effect of membrane irradiation for 1, 2 and 4 min in the presence of 5-AC (200 nM) on [^{3}H]citalopram binding was examined. In the presence of 5-AC, irradiation (1, 2 and 4 min) did not produce a significant reduction in the Bmax for [^{3}H]citalopram binding when compared with that for control membranes. However membrane irradiation for 4 min in the absence of drugs produced a significant 27% reduction in specific [^{3}H]citalopram binding when compared with that for control membranes, indicating that 4 min irradiation directly affected the membranes.

The effect of repeated U.V. irradiation (2 min x 2) on [^{3}H]citalopram binding was therefore examined. In the absence of 5-AC, the Bmax and K_D values of [^{3}H]citalopram binding to irradiated membranes were not significantly different from those for control membranes, indicating that the repeated irradiation did not directly affect the membranes. In the presence of 5-AC (200 nM), repeated irradiation showed a

significant reduction (20%) in the Bmax when compared with that for control membranes. Unlike the results with 400 W, the extent of the reduction in Bmax for [^{3}H]citalopram binding was similar and reproducible between experiments. The K_D value (0.99 nM) for non-irradiated membranes in the presence of 5-AC was significantly higher than that for control membranes (0.52 nM), but the K_D value (0.66 nM) for non-irradiated membranes in the presence of UV-5-AC (decomposed 5-AC by 2 min irradiation) was not different from that for control membranes. These results indicate that either UV-5-AC can be removed from the membranes by the washing procedure or because of its 9-fold lower affinity (Ki = 14.25 nM) than 5-AC (Ki = 1.65 nM) the amount remaining is not sufficient to significantly inhibit binding. However, despite the same extensive washing, 5-AC remaining in the membranes was sufficient to produce a 2-fold increase in the apparent K_D value for [^{3}H]citalopram. For this shift in K_D value, it can be calculated that approximately 1 nM residual 5-AC remained in the membranes. In the presence of 5-AC, repeated irradiation did not produce an alteration of the K_D value when compared with that for control membranes, suggesting that 5-AC in the membrane suspensions was decomposed into UV-5-AC by repeated 2 min irradiations. Thus it is concluded that 5-AC can produce a covalent bond at the [^{3}H]citalopram binding sites by repeated U.V. irradiation with 15 W. However, only about 20% of the [^{3}H]citalopram binding sites could be labelled despite the use of 5-AC at a concentration that completely inhibits specific [^{3}H]citalopram binding to rat cortical membranes and showed therefore saturable all the sites prior to exposure to irradiation.

Until recently many studies have been carried out using the arylazide compounds as photoaffinity ligands. For example, the use of azidoclebopride (a photoaffinity ligand for D_2 dopamine receptors) at a saturating concentration produced about a 60% reduction of the Bmax value for [^{3}H]spiperone binding to canine brain membranes (Niznik et al., 1984) and the irreversible binding of a saturating concentration of the azido-adenosine analogue R-AHPIA, to A_1 adenosine receptors has been indicated by a reduction in the Bmax (40%) for [^{3}H]N6-phenylisopropyl-adenosine binding to rat brain membranes (Klots et al., 1985). These reports could be examples of successful photoaffinity ligands. Thus the predictable photochemistry of aryl azides, and the highly reactive nature of the nitrenes have made the procedure popular. The aryl-substituted nitrenes give the highest efficiencies which is defined as the probability of a reversibly bound ligand becoming irreversibly bound, though on average this is still only 10% (Cavalla and Neff, 1985). The low efficiency of 5-AC-induced covalent modification may be due to the preferential reaction of nitrene with solvent rather than with the binding site (Hess et al., 1983; Niznik et al., 1984). As one of the reasons for low efficiency, it is known that the labelling bond of aryl azide photoaffinity ligands is sometimes unstable (Cavalla and Neff, 1985). In addition, the position of the photosensitive function, an azido group, on the chemical structure of the parent compound might be crucial for the determining the efficiency of a photoaffinity ligand. In this respect the 4'-azido-citalopram analogue which is predicted to have high affinity for the 5-HT transporter based on data with 4'-cyano-citalopram reported by Bigler et al. (1977)

(see introduction to this chapter) may be a possible alternative to 5-AC as a photoaffinity ligand. In conclusion, 5-AC can bind irreversibly to 20 % and up to 70 % of the [^{3}H]citalopram binding sites using repeated U.V. irradiations with 15 W (254 nm) and 400 W (365 nm) respectively. Therefore 5-AC, tritium labelled to a high specific activity, may provide a suitable photoaffinity ligand which could be used to isolate and characterise the native 5-HT transporter protein.

As a candidate for a SPET ligand for the 5-HT transporter, the 5-iodo analogue of citalopram (5-IC) was synthesised. 5-IC was 5.8-fold less potent than citalopram as an inhibitor of [^{3}H]5-HT uptake into rat cortical synaptosomes with a Ki value of 11.5 nM. As an inhibitor of [^{3}H]paroxetine and [^{3}H]citalopram binding to rat cortical membranes, 5-IC was only 1.9- and 3.1-fold less potent than citalopram (Ki : 4.0 nM and 2.9 nM respectively). These results suggest that 5-IC has a high affinity for the 5-HT transporter sites and might be a potential SPET ligand.

For further characterisation of 5-IC, a study using radio-iodinated 5-IC ([^{125}I]5-IC) was carried out. For the time course for [^{125}I]5-IC binding to rat cortical membranes, equilibrium was reached at 60 min, binding being slower than that for [^{3}H]citalopram. Binding of [^{125}I]5-IC to rat cortical membranes was inhibited by 5-IC (K_D = 1.87 nM), paroxetine (Ki = 0.12 nM), citalopram (Ki = 1.42 nM) and 5-HT (Ki = 248 nM). These Ki values corresponded well with data from the pharmacology of [^{3}H]citalopram and [^{3}H]paroxetine binding. As shown in fig.3.22, there was a good correlation between the potency of various

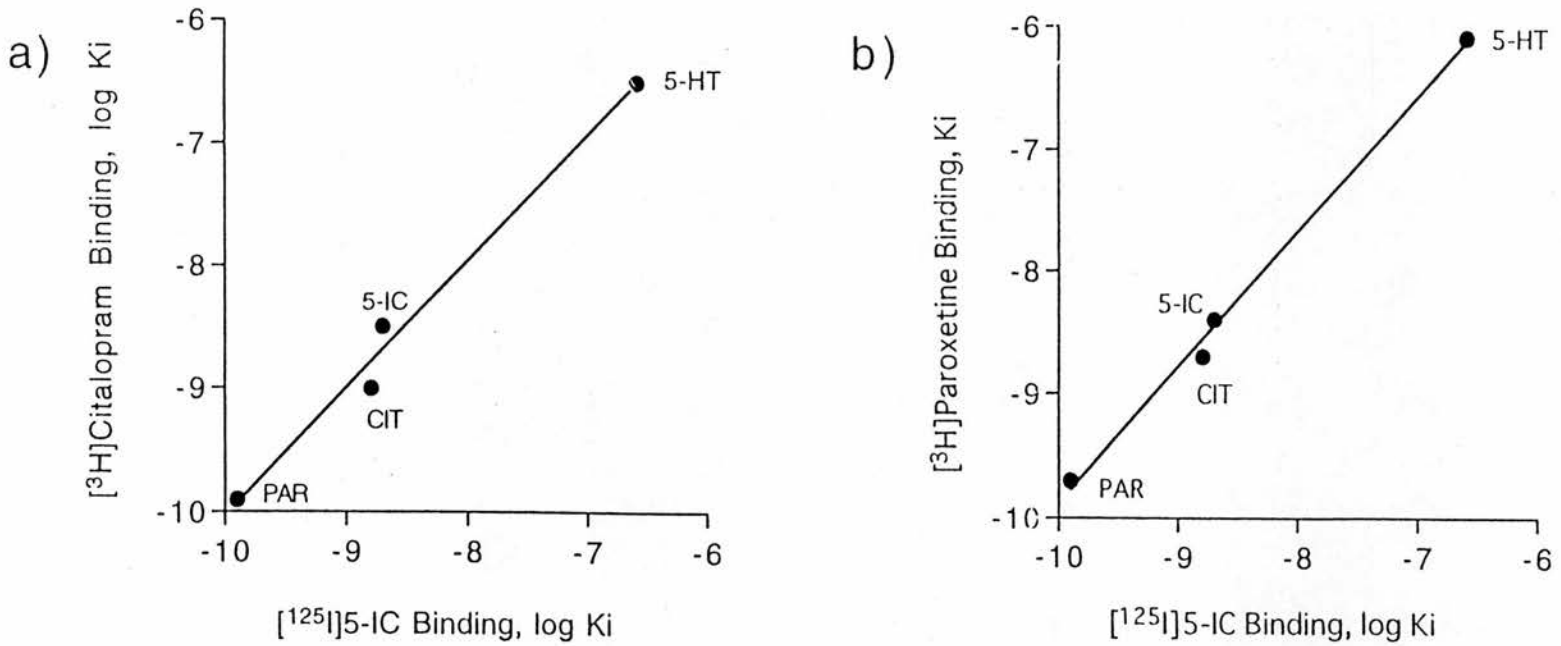


Fig. 3.22 Correlation between a) potency for inhibition of [125I]5-IC binding and potency for inhibition of [3H]citalopram binding, b) potency for inhibition of [125I]5-IC binding and potency for inhibition of [3H]paroxetine binding. The Ki values for inhibition of [125I]5-IC, [3H]citalopram and [3H]paroxetine binding to rat cortical membranes by the various drugs are taken from tables 3.2, 3.10 and 3.11. The correlation coefficient (r) was 0.994 for a) and 0.998 for b). The slope value was 1.04 for a) and 1.03 for b). PAR : paroxetine, CIT : citalopram, 5-IC : 5-iodo-citalopram.

drugs to inhibit [^{125}I]5-IC and [^{3}H]citalopram or [^{3}H]paroxetine binding. Their correlations for 4 drugs with r values of 0.994 (slope = 1.04, $P < 0.01$, [^{125}I]5-IC binding vs [^{3}H]citalopram binding) and 0.998 (slope = 1.03, $P < 0.01$, [^{125}I]5-IC binding vs [^{3}H]paroxetine binding), indicate that [^{125}I]5-IC, like [^{3}H]citalopram, is a suitable ligand for the 5-HT transporter. The Bmax value for [^{125}I]5-IC binding was not significantly different from those for [^{3}H]citalopram binding ($P = 0.157$) and [^{3}H]paroxetine binding ($P = 0.064$), suggesting that it was labelling the same population of binding sites.

The ability of [^{125}I]5-IC to label the 5-HT transporter sites in rat brain in vivo was examined. After injection of [^{125}I]5-IC, significant accumulation of radioactivity into rat whole brain was observed, indicating that 5-IC can penetrate the blood-brain barrier. The radioactivity increase with time in the liver may reflect the accumulation of metabolites of [^{125}I]5-IC. In addition, the relatively low radioactivity in the thyroid (maximum value : 0.082% of injected [^{125}I]5-IC at 1 hr post-injection) might indicate resistance to in vivo deiodination of [^{125}I]5-IC. In brain regions, at 30 min, the radioactivity concentration was similar in all brain regions but at all other time points the radioactivity concentrations in the cerebellum was lower in comparison with other 5 brain regions. In order to make accurate measurements of radioactivity in each brain region and examine the effect of pretreatment with unlabelled drug (paroxetine) on the brain regional distribution of [^{125}I]5-IC, 2 hr was chosen as a time point after injection, judging from the dispersion of data from 2 animals and sufficient radioactivity in each brain region for accurate measurements (Table 3.14,

Fig.3.19). After 2 hr of [^{125}I]5-IC injection, the brain regional distribution showed lowest radioactivity in the cerebellum (0.073 %D/g) compared with other brain regions (0.096 - 0.115 %D/g). In addition, the radioactivity in the cerebellum 2hr after [^{125}I]5-IC administration was not reduced in rats pretreated with paroxetine (1 mg/kg, i.v.) whereas a reduction of radioactivity by paroxetine was seen in other brain regions. Corresponding results to the present study have been shown using in vivo [^{3}H]cyanoimipramine and [^{3}H]paroxetine binding in animals (Wolfe et al., 1987; Scheffel and Hartig, 1989; Scheffel and Ricaurte, 1990). The radioactivity in hypothalamus and cortex after intravenous injection of [^{3}H]cyanoimipramine was reduced in rats pretreated with chlorimipramine, but no effect of chlorimipramine pretreatment was seen in the cerebellum (Wolf et al., 1987). Pretreatment with 5-HT uptake inhibitors such as citalopram and sertraline did not reduce the radioactivity in cerebellum after intravenous [^{3}H]paroxetine injection using mice or rats (Scheffel and Hartig, 1989; Scheffel and Ricaurte, 1990). It was considered that the radioactivity in the cerebellum represented nonspecific binding and specific binding was calculated by subtracting the radioactivity in the cerebellum from that in other brain regions. This hypothesis could be supported from in vitro autoradiographic studies using [^{3}H]paroxetine or [^{3}H]citalopram which showed that the cerebellum has a relatively low number of 5-HT uptake sites (DeSouza and Kuyatt, 1987; D'Amato et al., 1987a; Hrdina et al., 1989). As shown in table 3.16, the specific binding (%D/g in brain region - %D/g in cerebellum) in each brain region at 2hr after [^{125}I]5-IC injection was

calculated. When it was calculated by (individual brain region - average of control and paroxetine treated cerebellum (n=6)), there was no significant difference between control and paroxetine pretreatment because of the variation between animals. However when it was calculated by (individual brain region - individual cerebellum from corresponding animal), there was a significant difference between control and paroxetine pretreatment. The second way is valid because of analysis using matched tissue data. The highest level of specific [^{125}I]5-IC binding was observed in the brainstem (0.042 %D/g) and midbrain (0.037 %D/g), areas which are known to be rich in serotonergic neurons and consistent with previous reports using in vitro autoradiography with [^{3}H]paroxetine or [^{3}H]citalopram (DeSouza and Kuyatt, 1987; D'Amato et al., 1987a; Hrdina et al., 1989). As shown in table 3.16 and fig.3.20, pretreatment of paroxetine caused a significant reduction in specific [^{125}I]5-IC binding in all brain regions 2hr postinjection. The greatest reductions were observed in the midbrain (84.4%), rostral cortex (78.1%) and caudal cortex (63.2%). Smaller reductions occurred in the brainstem (49.8%) and hippocampus (34.8%). The reason for the brain regional variation in inhibitory effect of paroxetine on the specific [^{125}I]5-IC binding is not clear but similar results were reported by Scheffel and Ricaurte (1990). According to their report, pretreatment with citalopram or sertraline reduces specific in vivo [^{3}H]paroxetine binding in rat brain, but there are differences in the extent of reduction of specific [^{3}H]paroxetine binding between brain regions. For example, the greatest reductions by citalopram (3.2 mg/kg, i.v.) were observed in the superior colliculus (76%) and hypothalamus (69%), whereas the

reduction in the hippocampus (25%) was smallest. More recently, Hume et al. (1991) also reported the brain regional variation in inhibitory effects of paroxetine (2 mg/kg, i.v.) and citalopram (2 mg/kg, i.v.) on in vivo [^{3}H]citalopram binding in rats. These phenomena may indicate that a change in brain regional delivery of radioligand occurs by a pharmacologically active dose of drug (in the present experiments, 1 mg/kg paroxetine (Scheffel and Hartig, 1989)).

As pointed out by Scheffel and Hartig (1989), some requisites for a successful SPET ligand include good penetration across the blood-brain barrier, slow dissociation of the ligand from the binding site, a high ratio of specific to non-specific binding, and no rapid metabolic conversion of the tracer before its binding to the appropriate site. In the present study, [^{125}I]5-IC penetrated the blood-brain barrier and its peak radioactivity in whole brain was about 0.2 %D/g at 30-60 min after injection. In addition, the clearance of [^{125}I]5-IC in whole brain was demonstrated to be relatively slow and its half-life was greater than 90 min. This clearance pattern was similar in each brain region except cerebellum ; clearance of radioactivity from this brain region was faster than that from other brain regions. 2hr post-injection of [^{125}I]5-IC, the amount of radioactivity in each brain region except for cerebellum was approximately 0.1 %D/g and a clear difference ($P < 0.01$) in radioactivity was observed in comparison with cerebellum (0.07 %D/g). These data suggest that 2hr post-injection is a suitable time point for studying specific [^{125}I]5-IC binding in rat brain regions. Gibson et al. (1984) has reported that about 0.1 %D/g in rat caudate/putamen using [^{125}I]-4IQNB is a sufficient

level of radioactivity to provide SPET images of the distribution of muscarinic ACh receptors in human brain. Therefore, the radioactivity (0.1 %D/g) in brain regions obtained from the present study might be a sufficient amount for SPET studies in man. Recently, in vivo binding in rat brain using [^{3}H]citalopram and [^{11}C]citalopram was demonstrated by Hume et al. (1991). For both ligands, the relatively large nonspecific labelling in cerebellum results in only a relatively small proportion of specific binding, the ratios for brain regions to cerebellum at 1hr after injection being 1.23, 1.39 (frontal cortex), 1.17, 1.40 (thalamus) and 1.22, 1.32 (hippocampus) for [^{3}H] and [^{11}C] labelled citalopram binding, respectively. These values correspond well with the results obtained in the present work. However, it should be noted that they also showed that the single biologically active (+)enantiomer of [^{11}C]citalopram improved the ratios of radioactivity in brain regions relative to cerebellum the ratios being 1.68 (frontal cortex), 1.95 (thalamus) and 1.55 (hippocampus). Thus use of the (+)enantiomer of [^{125}I]5-IC might be expected to improve the ratios of specific to non-specific binding. Additionally, it has been pointed out that higher ratios may be obtained in human brain as the distribution of the 5-HT uptake sites appear to be more heterogeneous in human brain compared with rat brain (Wolfe et al, 1987).

The results from the present work and information from other reports indicate that [^{125}I]5-IC and, possibly [^{125}I](+)5-IC, is a potential SPET ligand for the 5-HT transporter in human brain. Such a SPET ligand would be valuable for monitoring neuropsychiatric diseases such as depression and for detecting

and evaluating damage of serotonergic neurons produced by potent neurotoxins such as MDMA. On the other hand, studies using other 5-HT uptake inhibitors as a prototype SPET ligands will yield useful information as to whether they are more suitable as prototype SPET ligands for the 5-HT transporter. As reported by Sheffel et al. (1990), the selective 5-HT uptake blocker fluoxetine does not appear to be a suitable SPET ligand candidate. The mouse brain regional distribution of [^{11}C]fluoxetine at 60 min after i.v. injection was not proportional to 5-HT uptake site densities unlike that of [^{11}C]citalopram. Although pretreatment with paroxetine (1mg/kg) significantly inhibited specific [^{11}C]citalopram binding, no inhibition of [^{11}C]fluoxetine binding was observed. Recently, a radioiodinated analogue of 6-nitroquipazine ([^{125}I]5-iodo-6-nitroquipazine) has been evaluated its in vitro and in vivo binding properties in rat brain (Mathis et al., 1993; Biegon et al., 1993). In vitro binding experiments in rat brain membranes demonstrated that [^{125}I]5-iodo-6-nitroquipazine is highly selective and has extremely high affinity (K_D = 23 pM) for 5-HT uptake sites (Mathis et al., 1993). In vivo studies using [^{125}I]5-iodo-6-nitroquipazine in rats have shown rapid and efficient penetration of the blood-brain barrier, and regional brain distribution correlated with the known distribution of serotonergic uptake sites and terminals (Biegon et al., 1993). In addition, in vivo imaging studies in non-human primates using SPET with [^{123}I]-labelled 5-iodo-6-nitroquipazine demonstrated that this tracer rapidly penetrates brain and yields good target-to-background ratios (e.g. brainstem to cerebellar ratio : > 2 by 8 hr after injection), indicating a promising compound for

imaging the serotonergic reuptake site in primate brain (Jagust et al., 1993). Finally taking data of in vivo [^{3}H]paroxetine binding using mice and rats into consideration, paroxetine may be expected to provide a useful SPET ligand candidate for the 5-HT transporter especially since it has very high affinity. However, there is no evidence from results shown usinadiochemical concentration of 10 mCi/ml in ethyl acetate. A batch of the crude material (200 mCi) was delivered to the Department of Pharmacology, University of Edinburgh and stored in 0.5 ml aliquots (10 mCi/ml) under liquid nitrogen prior to purification. For purification of [^{3}H](+)FR, after removal of ethyl acetate, 4.02 mCi of crude [^{3}H](+)FR was dissolved

CHAPTER 4

BINDING OF 5-HT$_{1A}$ RECEPTOR AND 5-HT TRANSPORTER LIGANDS IN RAT CORTEX AND HIPPOCAMPUS FOLLOWING CHOLINERGIC AND SEROTONERGIC LESION

4.1 INTRODUCTION

In Alzheimer's disease (AD) characterised by progressive deterioration of cognitive functions, neuropathological examination of the brains disclose characteristic abnormalities such as neuritic plaques which consist of abnormal neurites, and neurofibrillary tangles including bundles of paired helical filaments (Selkoe et al., 1982). The density of neuritic plaques in the cortex of AD patients correlates both with the severity of their cognitive defects and reductions in the activity of choline acetyltransferase (ChAT), the enzyme that synthesises acetylcholine (ACh) (Perry et al., 1978). These observations indicate that cortical cholinergic activity is correlated with the degree of dementia. This cholinergic deficiency in the cerebral cortex is thought to be due to a loss of neurons in the nucleus basalis of Meynert (nbM) which projects to the cerebral cortex (Coyle et al., 1983). In addition to the cholinergic deficiency, neurochemical studies have demonstrated that serotonergic dysfunction also occurs in AD (Bowen et al., 1983; Cross, 1988). In the brain of AD, it has been shown that 5-HT_1 and 5-HT_2 receptors are reduced using [^{3}H]5-HT (5-HT_1) and [^{3}H]ketanserin (5-HT_2) binding. More recently, Middlemiss et al. (1986) showed that a loss of [^{3}H]8-OH-DPAT binding sites (5-HT_{1A} receptor) is observed in frontal cortex from post-mortem AD brain compared with normal brain. In addition, the uptake of [^{3}H]5-HT and the binding of [^{3}H]imipramine are also significantly reduced (Bowen et al., 1983). D'Amato et al. (1987b) showed that a significant reduction of [^{3}H]citalopram binding to the 5-HT uptake sites without reductions of catecholaminergic markers occurs in frontal cortex of AD brain. These results suggest that 5-HT

uptake sites as well as 5-HT receptors are reduced in AD .

In order to study the role of the cholinergic system in learning and memory by pharmacological means, animal models with some of the neurochemical abnormalities present in AD have been developed. Ibotenate is often used to destroy the nbM in animals (Johnston et al., 1981; Wenk et al., 1984). The nbM lesioned rats have memory impairment which can be reversed pharmacologically with the ACh esterase inhibitor, physostigmine (Haroutunian et al., 1986), indicating that drugs that enhance central cholinergic activity can overcome the behavioural deficits produced by an nbM lesion. Recently, it has been shown that administration of the serotonergic depleting agent p-chloroamphetamine to nbM lesioned rats blocks the memory-enhancing effects of physostigmine (Santucci et al., 1989). This result may suggest that the serotonergic and cholinergic systems interact on a functional level in memory processing. Cross and Deakin (1985) observed a significant loss of cortical 5-HT_1 receptors using nbM lesioned rats with a large decrease in ChAT activity, indicating that a proportion of cortical 5-HT_1 receptors may be present on cholinergic terminals in the frontal cortex.

The aim of the work presented in this chapter was to investigate whether 5-HT_{1A} binding sites and 5-HT uptake sites are located on cholinergic nerve terminals in rat frontal cortex using selective neurotoxic lesion models. To destroy the cholinergic innervation of the cortex, two separate neurotoxins, ibotenate and AMPA, were injected directly into the nbM. AMPA infusions destroy more ChAT-immunoreactive neurons and cause greater reductions in cortical ChAT activity than ibotenate

infusions (Page et al.,1991). In addition, the location of 5-HT$_{1A}$ binding sites and 5-HT uptake sites in the frontal cortex and hippocampus was investigated in lesioned rats by intra-ventricular injection of the 5-HT neurotoxin 5,7-dihydroxy-tryptamine (5,7-DHT) to destroy 5-HT terminals. After producing the lesion with the neurotoxins, the binding site density and affinity for the 5-HT$_{1A}$ and 5-HT uptake sites were examined using [^{3}H]8-OH-DPAT and [^{3}H]citalopram binding, respectively.

4.2 METHODS

For these studies, surgery and stereotaxic injection of neurotoxins was carried out by Dr.S.P.Butcher in the Department of Pharmacology, University of Edinburgh.

4.2.1 Ibotenate and AMPA Lesions

Cholinergic neurones in the nbM were destroyed by direct injection of either ibotenate or AMPA according to the method of Wenk et al. (1984). Male Sprague Dawley rats (280-300 g) were allowed access to food and water ad libitum prior to the experiments. Under anaesthesia with pentobarbitone (60 mg/kg, i.p.), rats were placed in a Kopf stereotaxic frame (nose bar = 0.2 mm). Burr holes were drilled above nbM (AP -0.9 mm, ML ±2.6 mm) and 0.5 µl of either ibotenate (25 nmoles in 0.1 M phosphate buffered saline, pH 7.4) or AMPA (7.5 nmoles in 0.1 M phosphate buffered saline, pH 7.4) was injected over a 5 min period (DV -6.8 mm). The needle was left in place for 5 min before removal. Seven days after surgery, the rats were sacrified by decapitation and their brains were removed and

rapidly dissected on ice. The frontal cortex and hippocampus were isolated and stored at -20°C either for binding studies or for determination of ChAT activity. Control values were obtained from tissue samples derived from the hemisphere contralateral to the injection site with lesion data being obtained from the ipsilateral hemisphere.

4.2.2 ChAT Activity Assay

ChAT activity was determined according to the method of Fonnum (1975). Rats were killed and the cortices and hippocampi were dissected and homogenised in 20 vols of buffer (0.1% Triton X-100 in 1mM EDTA (pH 7.0)). The homogenates were diluted to 200 vols in the same buffer, and 20 µl aliquots were then added to 20 µl of incubation medium (50 mM NaH_2PO_4 (pH 7.4), 10 mM choline chloride, 0.1 mM eserine, 0.9 mg/ml albumin, 300 mM NaCl, 10 mM EDTA, 0.2 mM [^{14}C]acetyl-CoA). This mixture was incubated at 37°C for 20 min and the reaction was stopped by flushing the reaction medium into scintillation vials containig 10 ml scintillant (PPO in toluene) using 7 ml of extraction medium (5 ml of 10 mM NaH_2PO_4 (pH 7.4), 0.18 mg unlabelled ACh chloride, 37.4 mg sodium tetraphenylboron and 2 ml acetonitrile). The vials were gently inverted and radioactivity was determined after 60 min.

4.2.3 5,7-DHT Lesion

Male Sprague Dawley rats (280-300 g) were anaesthetised with pentobarbitone (60 mg/kg, i.p.) and placed in a Kopf stereotaxic frame. Thirty minutes after i.p. injection of 25 mg/kg desmethylimipramine to protect noradrenergic

neurones, 5,7-DHT (150 µg in 20 µl isotonic saline containing 0.1% ascorbic acid) was infused over 2 min into the lateral ventricle (AP -1.3 mm, ML -1.7 mm). The needle was left in place for a further 2 min before removal. Two weeks after surgery, the rats were sacrified by decapitation and their brains were removed and rapidly dissected at 4°C. The frontal cortex and hippocampus were isolated and stored at -20°C either for binding studies or for neurochemical assessment of the lesion by HPLC determination of 5-HT content. Values for control (untreated) and lesioned tissues are derived from separate groups of animals.

4.2.4 Measurement of Tissue 5-HT Concentration

Rats were killed and the cortices and hippocampi were dissected out, frozen on dry ice and stored at - 70°C. Tissues were weighed and homogenised in 0.6 M perchloric acid. Samples were centrifuged at 10,000g for 2 min at 4°C, and 20 µl aliquots of the supernatant were injected directly onto the HPLC. The 5-HT concentration in the supernatant was measured with electrochemical detection (BAS 4B; +0.7 V compared with a Ag/AgCl reference electrode) after separation by reversed-phase HPLC. Samples (20 µl) were chromatographed using a C18 Ultrasphere column (250 x 4.6 mm) and eluted with a mobile phase at a flow rate of 1 ml/min. As a mobile phase, 100 mM citrate/acetate buffer (pH 5.2 adjusted using NaOH) containing 100 µg/ml octanesulphonic acid, 5% methanol and 2% tetrahydrofuran was used. The system was calibrated by injection of 2 ng of 5-HT and the peak was identified by comparison of retention time.

4.2.5 Preparation of Membranes for Binding Assays

For binding experiments, individual hippocampi and frontal cortices were thawed at room temperature then homogenised in 40 vols of ice cold 50 mM Tris-HCl buffer (pH 7.4) and centrifuged at 30,000g at 4°C for 10 min. The pellet was washed 3 times by resuspension in 40 vols of Tris buffer and centrifugation, then resuspended in buffer and incubated at 37°C for 10 min to remove endogenous 5-HT. After a final centrifugation, the membranes were resuspended in 8 vols of Tris buffer and stored at -20°C until required. On the day of binding experiments, the membrane suspension was thawed at room temperature and diluted with Tris buffer; cortices 300 vols, hippocampi 500 vols.

4.2.6 [^{3}H]8-OH-DPAT Binding to Rat Cortical and Hippocampal Membranes

Binding of [^{3}H]8-OH-DPAT was carried out by preincubating duplicate 0.5 ml aliquots of membrane suspension with various concentrations of unlabelled 8-OH-DPAT or 50 mM Tris-HCl buffer containing $MgSO_4$ (final concentration 5 mM), pH7.4 (control) for 2 min at 25°C before addition of [^{3}H]8-OH-DPAT (final concentration 0.5 nM) to give a final assay volume of 1 ml. Samples were vortexed briefly then incubated at 25°C for 30 min. Incubation was terminated at room temperature by rapid filtration under vacuum on a Brandel Cell Harvester using Whatman GF/B filters then washed twice with 5 ml Tris buffer. The filters were transferred to scintillation vials and 100 µl of formic acid (100%) added to digest the membrane protein followed 10 min later by addition of 4 ml of scintillation fluid

(Packard Emulsifier Safe). The radioactivity trapped on the filter was measured in a Packard 1900CA liquid scintillation analyser using automatic quench correction. 10 μM 5-HT was used to define specific binding. Quadruplicate 100 μl samples of [^{3}H]8-OH-DPAT as standards were counted in order for the exact ligand concentration to be calculated for individual experiments.

4.2.7 [^{3}H]Citalopram Binding to Rat Cortical and Hippocampal Membranes

[^{3}H]Citalopram and [^{3}H]8-OH-DPAT bindings were carried out in parallel using the same membrane preparations from control and lesioned rats as described in section 4.2.5. Therefore the assay was a modification of the method described in section 3.2.4 since no NaCl or KCl was present in the final membrane preparation. Instead NaCl and KCl was added directly to the assay tube to give a final concentration of 120 mM NaCl and 5 mM KCl. Thus binding was carried out by preincubating duplicate 500 μl aliquots of membrane suspension and 200 μl of Tris-HCl buffer containing 600 mM NaCl and 25 mM KCl with 10 concentrations of unlabelled citalopram or Tris buffer (control) for 2 min at 25°C before addition of [^{3}H]citalopram (final concentration 0.5 nM) to give a final assay volume of 1 ml. Samples were incubated at 25°C for 60 min. Incubation was terminated by rapid filtration on a Brandel Cell Harvester (Whatman GF/B filter, pretreated with 0.05% polyethyleneimine) followed by two washes with 5 ml Tris buffer. The filters were transferred to scintillation vials, and 4 ml of scintillation fluid (Packard Emulsifier Safe) was added to each vial 10 min after addition of 100% formic acid (100 μl) to digest the membrane.

The radioactivity trapped on the filter was measured in a Packard 1900CA liquid scintillation analyser using automatic quench correction. 10 μM unlabelled citalopram was used to define specific binding. In preliminary experiments this method for [^{3}H]citalopram binding was compared directly with the method described in section 3.2.4. When NaCl and KCl were added to the assay tubes, K_D and Bmax values for [^{3}H]citalopram binding were 0.9 nM and 0.9 pmol/mg protein respectively. Using the method described in section 3.2.4, K_D and Bmax values for [^{3}H]citalopram binding were 0.8 nM and 0.8 pmol/mg protein respectively. Therefore virtually identical results were obtained allowing a single membrane suspension to be used as the tissue source for both the [^{3}H]8-OH-DPAT and [^{3}H]citalopram assays.

4.2.8 Protein Assay

Protein assay was carried out as described in section 3.2.7. In brief, the reagent (2.5 ml) containing phosphoric acid and Coomassie Brilliant Blue was added to 100 μl samples and 20 min later the optical density of sample was read at 595 nm on a spectrophotometer. The protein concentration of membrane samples was calculated from the standard curve using bovine serum albumin (25-200 μg/ml).

4.2.9 Data Analysis

Data for the binding of [^{3}H]8-OH-DPAT and [^{3}H]citalopram were analysed by least squares fit to the logistic expression :

$$Y = MX^P / (X^P + IC_{50})$$

Ki values were calculated from the equation :

$$Ki = IC_{50} / (1 + L/K_D)$$

where L is ligand concentration. Bmax value were calculated from the equation :

$$b = Bmax \cdot L / (L + K_D)$$

where b is bound ligand concentration. These kinetic variables together with neurochemical data (ChAT activity and 5-HT content) were determined for individual animals. Values from each control and lesion group were then combined to provide a mean ± S.E.M. and data subjected to statistical analysis using Wilcoxon signed rank test or a Mann Whitney U-test as appropriate.

4.2.10 Materials

[^{14}C]Acetyl CoA (specific activity : 60 mCi/mmol), [^{3}H]8-OH-DPAT (specific activity : 264 Ci/mmol) and [^{3}H]citalopram (specific activity : 50 Ci/mmol) were obtained from Amersham International. [^{3}H]8-OH-DPAT and [^{3}H]citalopram were diluted to 1 µM in glass distilled water and stored under liquid nitrogen in volumes sufficient for individual experiments. Ibotenate was obtained from Research Biochemicals Inc. and AMPA was obtained from Tocris Neuroamin. Other drugs and reagents were obtained from Sigma.

4.3 RESULTS

4.3.1 Effects of Ibotenate and AMPA Lesions on ChAT Activity

Seven days after nbM lesion by ibotenate and AMPA, cortical and hippocampal ChAT activity was measured. In experiments using both ibotenate and AMPA, large and significant reductions in ChAT activity were observed in frontal

cortex but not in hippocampus (Table 4.1). In the frontal cortex ipsilateral to the ibotenate injection site, a 49.8% reduction of ChAT activity (27.3 ± 3.1 nmol/mg protein/hr) was observed in comparison with that in the contralateral side (54.4 ± 3.9 nmol/mg protein/hr), whereas lesion by ibotenate did not affected ChAT activity in the hippocampus (control : 61.3 ± 5.8, lesion : 62.3 ± 5.2 nmol/mg protein/hr). AMPA lesion reduced the cortical ChAT activity by 67.5 % (control : 46.5 ± 4.1, lesion : 15.1 ±1.7 nmol/mg protein/hr). In contrast, the hippocampal ChAT activity was not affected by AMPA injection (control : 56.5 ± 5.5, lesion : 55.9 ± 5.3 nmol/mg protein/hr).

4.3.2 Effect of 5,7-DHT Lesions on 5-HT Levels

Two weeks after i.c.v. injection of 5,7-DHT, the 5-HT levels in the lesioned rats were measured using HPLC and compared with those in sham controls (Table 4.2). 5-HT levels in the frontal cortex and hippocampus of 5,7-DHT lesioned animals were 1.23 ± 0.13 and 0.68 ± 0.09 pmol/mg wet weight respectively. Corresponding values in untreated animals were 5.53 ± 0.43 and 4.1 ± 0.43 pmol/mg wet weight. Thus 5-HT levels were reduced by 78% and 83% in the frontal cortex and hippocampus of lesioned animals.

4.3.3 Kinetic Characteristics of [^{3}H]8-OH-DPAT Binding to Rat Cortical and Hippocampal Membranes (Table 4.3)

In unlesioned tissue of three separate sets of experiments (ibotenate, AMPA or 5,7-DHT lesion), the binding affinity (K_D) of [^{3}H]8-OH-DPAT in cortical membranes was 1.38 ± 0.09 nM, 1.21 ± 0.04 nM and 1.46 ± 0.12 nM respectively, whereas the K_D in

TABLE 4.1 : Effects of Basal Forebrain Lesions on Cortical and Hippocampal ChAT Activity

	ChAT activity (nmol/mg protein/hr)	
	Control	Lesion
Ibotenate		
Cortex	54.4 ± 3.9	27.3 ± 3.1 ** (49.8)
Hippocampus	61.3 ± 5.8	62.3 ± 5.2
AMPA		
Cortex	46.5 ± 4.1	15.1 ± 1.7 ** (67.5)
Hippocampus	56.5 ± 5.5	55.9 ± 5.3

Choline acetyltransferase (ChAT) activity was determined according to the method of Fonnum (1975). Values are mean ± S.E.M. from 6 animals. Control and lesion data were obtained from the unlesioned and lesioned hemispheres of the same rat. ** ; $P < 0.01$ compared with control. % reduction versus control was shown in parentheses.

TABLE 4.2 : Effect of 5,7-DHT Lesion on Cortical and Hippocampal 5-HT Content

	5-HT content (pmol/mg wet weight)	
	Control	Lesion
Cortex	5.53 ± 0.43	1.23 ± 0.13 *** (78)
Hippocampus	4.10 ± 0.43	0.68 ± 0.09 *** (83)

5-HT levels in the cortex and hippocampus of unlesioned and 5,7-DHT injected (150 μg, i.c.v.) rats were measured by HPLC. Results are the mean ± S.E.M. from 6 animals. *** ; $P < 0.001$ compared with control. % reduction versus control was shown in parentheses.

TABLE 4.3 : Effects of Neuronal Lesions on [^{3}H]8-OH-DPAT Binding to Rat Brain Membranes

		[^{3}H]8-OH-DPAT binding	
		K_D (nM)	Bmax (pmol/mg protein)
Ibotenate lesion			
Cortex	Control	1.38 ± 0.09	0.45 ± 0.02
	Lesion	1.38 ± 0.06	0.52 ± 0.03
Hippocampus	Control	1.23 ± 0.12	1.83 ± 0.06
	Lesion	1.28 ± 0.08	1.85 ± 0.14
AMPA lesion			
Cortex	Control	1.21 ± 0.04	0.44 ± 0.06
	Lesion	1.19 ± 0.02	0.47 ± 0.03
Hippocampus	Control	1.03 ± 0.04	1.91 ± 0.21
	Lesion	0.96 ± 0.03	1.75 ± 0.19
5,7-DHT lesion			
Cortex	Control	1.46 ± 0.12	0.68 ± 0.04
	Lesion	1.42 ± 0.09	0.61 ± 0.07
Hippocampus	Control	1.73 ± 0.10	1.96 ± 0.12
	Lesion	1.50 ± 0.13	1.64 ± 0.12

Membranes were incubated with [^{3}H]8-OH-DPAT (0.5 nM) in the absence or presence of unlabelled 8-OH-DPAT for 30 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled 5-HT. Results are the mean ± S.E.M. from 6 independent samples being derived either from the same rat (for ibotenate and AMPA lesions, membranes were prepared from the lesioned and unlesioned hemispheres of the same brain) or from different animals (for 5,7-DHT lesions, membranes were prepared from different animals).

hippocampal membranes was 1.23 ± 0.12 nM, 1.03 ± 0.04 nM and 1.73 ± 0.10 nM respectively. The binding density (Bmax) in cortical membranes was 0.45 ± 0.02 pmol/mg protein (ibotenate), 0.44 ± 0.06 pmol/mg protein (AMPA) and 0.68 ± 0.04 pmol/mg protein (5,7-DHT). In hippocampal membranes, the Bmax was 1.83 ± 0.06 pmol/mg protein (ibotenate), 1.91 ± 0.21 pmol/mg protein (AMPA) and 1.96 ± 0.12 pmol/mg protein (5,7-DHT). Thus the Bmax in hippocampal membranes was greater than in cortical membranes in three separate sets of experiments.

4.3.4 Effects of Neuronal Lesions on [^{3}H]8-OH-DPAT Binding

a) Ibotenate lesion (Table 4.3)

In the frontal cortex and hippocampus contralateral to the ibotenate injection site, the binding affinity (K_D) of [^{3}H]8-OH-DPAT was 1.38 ± 0.09 nM and 1.23 ± 0.12 nM respectively. The K_D value in cortical and hippocampal membranes from ibotenate lesioned hemisphere was 1.38 ± 0.06 nM and 1.28 ± 0.08 nM respectively. Thus basal forebrain cholinergic lesions using ibotenate did not effect the binding affinity of [^{3}H]8-OH-DPAT. The [^{3}H]8-OH-DPAT binding site density (Bmax) in membranes from the unlesioned hemisphere was 0.45 ± 0.02 pmol/mg protein (cortex) and 1.83 ± 0.06 pmol/mg protein (hippocampus), while the Bmax in membranes from lesioned hemisphere was 0.52 ± 0.03 pmol/mg protein (cortex) and 1.85 ± 0.14 pmol/mg protein (hippocampus), suggesting that the ibotenate lesion did not affected the Bmax.

b) AMPA lesion (Table 4.3)

The K_D of [^{3}H]8-OH-DPAT in membranes from unlesioned hemisphere was 1.21 ± 0.04 nM (cortex) and 1.03 ± 0.04 nM (hippocampus), while the K_D in membranes prepared from lesioned hemisphere was 1.19 ± 0.02 nM (cortex) and 0.96 ± 0.03 nM (hippocampus). The Bmax of [^{3}H]8-OH-DPAT in membranes from the AMPA lesioned hemisphere was 0.47 ± 0.03 pmol/mg protein (cortex) and 1.75 ± 0.19pmol/mg protein (hippocampus). These values were not significantly different from those for unlesioned hemisphere (cortex : 0.44 ± 0.06 pmol/mg protein, hippocampus : 1.91 ± 0.21 pmol/mg protein). These results suggest that the K_D and Bmax values of [^{3}H]8-OH-DPAT were not affected by AMPA induced lesions.

c) 5,7-DHT lesion (Table 4.3)

There was no significant difference in the K_D values of [^{3}H]8-OH-DPAT in membranes between the 5,7-DHT lesioned animals (cortex : 1.42 ± 0.09 nM, hippocampus : 1.50 ± 0.13 nM) and unlesioned animals (cortex : 1.46 ± 0.12 nM, hippocampus : 1.73 ± 0.10 nM). The Bmax of [^{3}H]8-OH-DPAT in membranes from the 5,7-DHT lesioned animals was 0.61 ± 0.07 pmol/mg protein (cortex) and 1.64 ± 0.12 pmol/mg protein (hippocampus), while the Bmax in membranes from unlesioned animals was 0.68 ± 0.04 pmol/mg protein (cortex) and 1.96 ± 0.12 pmol/mg protein (hippocampus). These results suggest that the K_D and Bmax values of [^{3}H]8-OH-DPAT were not significantly affected by 5,7-DHT lesions.

4.3.5 Kinetic Characteristics of [^{3}H]Citalopram Binding to Rat Cortical and Hippocampal Membranes (Table 4.4)

In unlesioned tissue, the binding affinity (K_D) of [^{3}H]citalopram in cortical membranes was 0.85 ± 0.09 nM (ibotenate), 3.03 ± 0.28 nM (AMPA) and 2.26 ± 0.14 nM (5,7-DHT) in three separate sets of experiments. The K_D in hippocampal membranes was 1.22 ± 0.38 nM (ibotenate), 2.95 ± 0.28 nM (AMPA) and 0.66 ± 0.17 nM (5,7-DHT). The binding density (Bmax) in cortical membranes was 1.66 ± 0.15 pmol/mg protein (ibotenate), 1.77 ± 0.24 pmol/mg protein (AMPA) and 1.59 ± 0.07 pmol/mg protein (5,7-DHT). In hippocampal membranes, the Bmax was 1.34 ± 0.27 pmol/mg protein (ibotenate), 0.92 ± 0.16 pmol/mg protein (AMPA) and 0.78 ± 0.13 pmol/mg protein (5,7-DHT). The K_D or Bmax values varied from one set of experiment to another although the actual value within a set of experiments showed little variation (data not shown). The reasons underlying the discrepancy between the K_D or Bmax values between the three sets of experiments remain unclear. In all experiments, the Bmax in cortical membranes was greater than in hippocampal membranes.

4.3.6 Effects of Neuronal Lesions on [^{3}H]Citalopram Binding

a) Ibotenate lesion (Table 4.4)

The K_D of [^{3}H]citalopram in cortical and hippocampal membranes from ibotenate lesioned hemisphere was 0.80 ± 0.06 nM and 1.43 ± 0.47 nM respectively. These values were not significantly different from those for unlesioned hemisphere (cortex : 0.85 ± 0.09 nM, hippocampus : 1.22 ± 0.38 nM). The [^{3}H]citalopram binding site density (Bmax) in membranes from

TABLE 4.4 : Effects of Neuronal Lesions on [^{3}H]Citalopram Binding to Rat Brain Membranes

		[^{3}H]Citalopram binding	
		K_D (nM)	Bmax (pmol/mg protein)
Ibotenate lesion			
Cortex	Control	0.85 ± 0.09	1.66 ± 0.15
	Lesion	0.80 ± 0.06	1.33 ± 0.12
Hippocampus	Control	1.22 ± 0.38	1.34 ± 0.27
	Lesion	1.43 ± 0.47	1.37 ± 0.34
AMPA lesion			
Cortex	Control	3.03 ± 0.28	1.77 ± 0.24
	Lesion	3.34 ± 0.30	1.74 ± 0.10
Hippocampus	Control	2.95 ± 0.28	0.92 ± 0.16
	Lesion	3.76 ± 0.62	1.12 ± 0.15
5,7-DHT lesion			
Cortex	Control	2.26 ± 0.14	1.59 ± 0.07
	Lesion	3.03 ± 0.36	0.84 ± 0.11 ***
Hippocampus	Control	0.66 ± 0.17	0.78 ± 0.13
	Lesion	N.D.	N.D.

Membranes were incubated with [^{3}H]citalopram (0.4 nM) in the absence or presence of unlabelled citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Results are the mean ± S.E.M. from 6 independent samples being derived either from the same rat (for ibotenate and AMPA lesions, membranes were prepared from the lesioned and unlesioned hemispheres of the same brain) or from different animals (for 5,7-DHT lesions, membranes were prepared from different animals). *** ; $P < 0.001$ compared with control. N.D. ; binding was not detectable.

the unlesioned hemisphere was 1.66 ± 0.15 pmol/mg protein (cortex) and 1.34 ± 0.27 pmol/mg protein (hippocampus), while the Bmax in membranes from lesioned hemisphere was 1.33 ± 0.12 pmol/mg protein (cortex) and 1.37 ± 0.34 pmol/mg protein (hippocampus), suggesting that the ibotenate lesion did not affected the Bmax.

b) AMPA lesion (Table 4.4)

The K_D of [^{3}H]citalopram in membranes from unlesioned hemisphere was 3.03 ± 0.28 nM (cortex) and 2.95 ± 0.28 nM (hippocampus), while the K_D in membranes prepared from lesioned hemisphere was 3.34 ± 0.30 nM (cortex) and 3.76 ± 0.62 nM (hippocampus). The Bmax of [^{3}H]citalopram in membranes from the AMPA lesioned hemisphere was 1.74 ± 0.10 pmol/mg protein (cortex) and 1.12 ± 0.15 pmol/mg protein (hippocampus). These values were not significantly different from those for unlesioned hemisphere (cortex :1.77 ± 0.24 pmol/mg protein, hippocampus : 0.92 ± 0.16 pmol/mg protein). These results suggest that the K_D and Bmax values of [^{3}H]citalopram were not affected by AMPA induced lesions.

c) 5,7-DHT lesion (Table 4.4)

There was no significant difference in the K_D values of [^{3}H]citalopram in cortical membranes between the 5,7-DHT lesioned animals (3.03 ± 0.36 nM) and unlesioned animals (2.26 ± 0.14 nM). In the frontal cortex of the 5,7-DHT lesioned animals, a 47.2% reduction of the Bmax value of [^{3}H]citalopram (0.84 ± 0.11 pmol/mg protein) was observed in comparison with that for unlesioned animals (1.59 ± 0.07 pmol/mg protein). In

the hippocampus, [^{3}H]citalopram binding was almost undetectable in lesioned animals. Since it was not possible to analyse the data accurately and calculate K_D and Bmax values for [^{3}H]citalopram binding in hippocampus from lesioned animals due to the small amounts of specific binding, a direct comparison of the specific binding at a single ligand concentration of 0.5 nM is shown in table 4.5 for both hippocampus and frontal cortex. In the hippocampus, 5,7-DHT lesion reduced significantly the specific [^{3}H]citalopram binding by 87.4% (control; 308.3 ± 16.1 fmol/mg protein, lesion; 38.7 ± 6.7 fmol/mg protein) whereas a 59.5% reduction of specific binding was shown in the frontal cortex (control; 307.0 ± 19.7 fmol/mg protein, lesion; 124.3 ± 11.1 fmol/mg protein).

4.4 DISCUSSION

The cellular localisation of 5-HT$_{1A}$ binding sites and 5-HT uptake sites in the rat frontal cortex and hippocampus was examined using three different neurotoxin, ibotenate, AMPA and 5,7-DHT. The relevant binding sites were characterised in membrane binding assays which used [^{3}H]8-OH-DPAT to study the 5-HT$_{1A}$ binding site and [^{3}H]citalopram to identify the serotonin transport site.

In order to destroy cholinergic terminals, ibotenate and AMPA was injected into nbM which provides the major cholinergic projections to the cortex (Coyle et al., 1983). After lesions, ChAT activity which is a marker for cholinergic terminals was measured in the frontal cortex and hippocampus. Both neurotoxins reduced ChAT activity in frontal cortex but not in hippocampus, indicating that ibotenate and AMPA produced a

TABLE 4.5 : Effect of 5,7-DHT Lesion on Specific [^{3}H]Citalopram Binding

	Specific [^{3}H]citalopram binding (fmol/mg protein)	
	Control	Lesion
Cortex	307.0 ± 19.7	124.3 ± 11.1 *** (59.5)
Hippocampus	308.3 ± 16.1	38.7 ± 6.7 *** (87.4)

Membranes were incubated with [^{3}H]citalopram in the absence or presence of unlabelled citalopram for 60 min at 25°C. Non-specific binding was determined in the presence of 10 μM unlabelled citalopram. Specific [^{3}H]citalopram binding at a single ligand concentration of 0.5 nM was shown. Results are the mean ± S.E.M. (n = 6) from the cortex and hippocampus of unlesioned and 5,7-DHT injected (150μg, i.c.v.) rats. *** ; $P < 0.001$ compared with control. % reduction versus control was shown in parentheses.

selective destruction of cholinergic nerve terminals in frontal cortex. AMPA lesion produced a 67.5% reduction of ChAT activity in frontal cortex and this was significantly greater ($P < 0.01$) than the 49.8% reduction noted after ibotenate lesion. This result is consistent with previous observations by Page et al. (1991). They showed that AMPA- and ibotenate-induced lesions reduced cortical ChAT activity by 70% and 50% respectively and this difference was due to a less effective in destruction of cholinergic neurons by an ibotenate lesion than an AMPA lesion as shown by histological analysis.

Excitotoxic lesions of basal forebrain cholinergic neurones using either ibotenate or AMPA did not effect the binding affinity (K_D value) and density (Bmax value) for [^{3}H]8-OH-DPAT binding in either cortical or hippocampal membranes. These data suggest that the 5-HT_{1A} binding sites are not localised on the cortical nerve terminals of cholinergic neurones situated in the basal forebrain. However these data should be contrasted with the results of release experiments which suggest that 5-HT_{1A} receptor agonists increase cortical ACh release (Bianchi et al., 1990). This discrepancy is presumably due either to species differences between the two studies (guinea pigs were used in the release experiments) or to an action of the 5-HT_{1A} receptor agonist, which was administered systemically in the release experiment, on cholinergic cell bodies in the basal forebrain resulting in an alteration in cortical release of ACh. Previously Cross and Deakin (1985) have showed that the lesion of nbM by ibotenate produces a significant reduction in ChAT activity (56%) and the Bmax value (18%) for [^{3}H]5-HT binding in rat cortex, indicating that a proportion of 5-HT_1 receptors may

be associated with cholinergic terminals. However it is known that [^{3}H]5-HT binds to several distinct subtypes including 5-HT$_{1A,1B,1C}$ and $_{1D}$. 5-HT$_{1C}$ is now reclassified as the 5-HT$_{2C}$ receptor. It has been shown that relative contributions of four subtypes to total 5-HT$_1$ binding sites in rat cortex are 48% (5-HT$_{1A}$), 14% (5-HT$_{1B}$), 20% (5-HT$_{2C}$) and 18% (5-HT$_{1D}$) (Herrick-Davis and Titeler, 1988). Judging from results in the present experiment, a loss of cortical 5-HT$_1$ receptors by ibotenate lesion might be due to the destruction of other 5-HT$_1$ receptors except for the 5-HT$_{1A}$ subtype. On the other hand, Middlemiss et al. (1986) have demonstrated a 48% loss of [^{3}H]8-OH-DPAT binding sites in the frontal cortex from patients with AD. The results in the present experiment indicate that a loss of cortical 5-HT$_{1A}$ receptors in AD is unlikely to be due to a loss of cholinergic terminals. However, the serotonin innervation in human frontal cortex might be different from that in rats. Indeed, Hoyer et al (1986a) have demonstrated that [^{3}H]8-OH-DPAT binding to the 5-HT$_{1A}$ receptor in human brain is dense in outer layers of the cerebral cortex whereas this is not the case in rats (Pazos and Palacios, 1985). Thus a loss of 5-HT$_{1A}$ receptors in AD may occurs especially in outer layers of the cerebral cortex. Recently it has been suggested that the degeneration of cortical glutamatergic neurones occurs in the brain from patients with AD (Francis et al., 1993). Endogenous 5-HT may regulate glutamate transmission at 5-HT$_{1A}$ receptors located on cortical glutamatergic neurones (Francis et al., 1993). Therefore a loss of 5-HT$_{1A}$ receptors in AD might be due to degeneration of cortical glutamatergic neurones.

The destruction of serotonergic neurons by 5,7-DHT reduced markedly and significantly the 5-HT levels in rat frontal cortex and hippocampus in comparison with those in unlesioned animals, indicating a loss of serotonergic terminals. Lesioning of serotonergic neurones had no effect on [^{3}H]8-OH-DPAT binding in the cortex and hippocampus. Previously, Hall et al. (1985) demonstrated that intracerebral 5,7-DHT injection produces a significant reduction (51%) in the Bmax value for [^{3}H]8-OH-DPAT binding in the striatum but not in the hippocampus or cerebral cortex. Thus data from the present study are in agreement with their findings.

Lesions of basal forebrain cholinergic neurones by ibotenate and AMPA did not effect the affinity or binding site density of [^{3}H]citalopram binding in either cortical or hippocampal membranes. These results suggest that the 5-HT uptake sites are not localised on cholinergic terminals in rat frontal cortex. Therefore the reduction of [^{3}H]citalopram binding sites observed in human frontal cortex with AD (D'Amato et al., 1987b) may not directly depend on a damage to cholinergic terminals. Serotonergic lesions produced a reduction of binding site density for [^{3}H]citalopram binding in both the cortical and hippocampal membranes. These results suggest that [^{3}H]citalopram can label the 5-HT transporter on serotonergic terminals in rat frontal cortex and hippocampus. However there was a marked difference in the extent of the reduction in the cortex and hippocampus. The density of [^{3}H]citalopram binding sites was almost abolished in the hippocampus whereas its reduction in the frontal cortex was only 47.2%. In the specific [^{3}H]citalopram binding, a 87.4% reduction was observed in the

hippocampus when compared with unlesioned animals, whereas that in the frontal cortex was 59.5%. It is possible that the difference in reduction of transporter sites for the two brain regions is due to lower penetration of the neurotoxin in the cortex. However this seems an unlikely explanation since 5-HT levels were reduced to a similar extent in frontal cortex (75.1%) and hippocampus (78.6%). The discrepancy may suggest that [^{3}H]citalopram labels a second population of binding sites in the frontal cortex that is not associated with serotonergic nerve terminals. However there is no evidence to suggest that two pharmacologically distinct [^{3}H]citalopram binding sites exist in rat brain. An alternative explanation is that the cellular distribution of the binding site in the cortex and hippocampus differs. 5-HT uptake sites have been demonstrated in primary astrocyte cultures as well as in neurones (Katz and Kimelberg, 1985). A differential contribution of glial cells and neurones to the pattern of binding in the two brain areas could explain these data because the neurotoxic effect of 5,7-DHT would only be expected to destroy the neuronal [^{3}H]citalopram binding sites. However Habert et al. (1985) showed that 5,7-DHT lesion produces a 90% reduction in [^{3}H]paroxetine binding to rat cortical membranes. In addition it has been reported that lesioning of the serotonergic neurons with p-chloroamphetamine causes a 90% reduction of [^{3}H]citalopram binding sites to rat cortical membranes (D'Amato et al., 1987a). Therefore the difference in the extent of the reduction of [^{3}H]citalopram binding sites in the cortex and hippocampus observed in the present study remain unclear.

In conclusion, the 5-HT_{1A} receptors are not localised on cholinergic terminals in the cortex, or serotonergic terminals in the cortex and hippocampus. The 5-HT transporter sites are located on terminals of serotonergic neurons in the cortex and hippocampus, but not on cholinergic terminals in the cortex.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Characterisation of the NMDA receptor antagonist, FR115427

The novel isoquinoline derivative FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride), is a novel NMDA antagonist and has antiischaemic and anticonvulsive effects in animals. Therefore, FR115427 ((+)FR) was characterised initially using in vitro [^{3}H]MK-801 binding to rat brain membranes. Subsequently, [^{3}H]FR115427 was synthesised, purified and then its in vivo distribution in rats was examined.

(+)FR inhibited [^{3}H]MK-801 binding to rat cortical synaptosomal membranes in the presence of added L-glutamate (10 μM) with a Ki value of about 40 nM and although it was 12-fold less potent than (+)MK-801, the data are consistent with (+)FR being a non-competitive NMDA antagonist. As observed with (+)MK-801, the affinity of (+)FR was 2.9-fold higher when 10 μM L-glutamate was present in the assay buffer. These results suggest that binding of glutamate to the NMDA receptor presumably causes a conformational change that facilitates the interaction of (+)FR with its binding site within the NMDA receptor ion channel. However the effect of L-glutamate was more apparent for (+)MK-801 than (+)FR, indicating that (+)FR and (+)MK-801 may not interact with the binding site in the NMDA receptor-ion channel in an identical manner. When compared with the corresponding (-)enantiomer, (+)FR and (+)MK-801 were respectively 98- and 4.2-fold more potent as inhibitors of [^{3}H]MK-801 binding to rat cortical synaptosomal membranes in the presence of added L-glutamate

(10 μM). These results suggest a more pronounced stereoselectivity for FR compared with MK-801. This difference is most likely due to the differences in the chemical structure of MK-801 and FR115427.

[^{3}H](+)FR synthesised by tritiation of (+)Cl-FR (mono-chlorinated aromatic precursor of (+)FR) was successfully purified by an open column method, and its purity confirmed by TLC and column chromatography. Intravenous injection of [^{3}H](+)FR resulted in a rapid accumulation of radioactivity in rat brain. Therefore [^{3}H](+)FR might be useful tool for in vivo imaging of the NMDA receptor-ion channel complex, especially under cerebral ischaemic conditions, although further detailed experiments will be required.

Development of photoaffinity and SPET ligands for the 5-HT transporter

For evaluation of citalopram analogues as photoaffinity and SPET ligands for the 5-HT transporter, assays for [^{3}H]5-HT uptake into rat cortical synaptosomes and the binding of [^{3}H]paroxetine and [^{3}H]citalopram to rat cortical membranes were established and evaluated. There was a good correlation between the potency of various drugs to inhibit [^{3}H]5-HT uptake, [^{3}H]paroxetine and [^{3}H]citalopram binding.

5-Azido-citalopram (5-AC) synthesised as a candidate for a photoaffinity ligand, had high affinity (Ki = 1.65 nM) for [^{3}H]citalopram binding sites and 5-AC was only 1.8-fold less potent than citalopram itself. In the presence of 200 nM, a concentration of 5-AC which can completely inhibit

[^{3}H]citalopram binding, repeated U.V. irradiation of rat cortical membranes with 15 W for 2 min produced a significant 20% reduction in the Bmax value for [^{3}H]citalopram binding compared with control, indicating formation of a covalent bond between 5-AC and [^{3}H]citalopram binding sites. Therefore, radiolabelled 5-AC may provide a tool for isolation and characterisation of the 5-HT transporter.

5-Iodo-citalopram (5-IC) synthesised as a candidate for a SPET ligand, was 5.8-fold less potent than citalopram as an inhibitor of [^{3}H]5-HT uptake into rat cortical synaptosomes with a Ki value of 11.5 nM. This compound had high affinity (Ki = 4.0 nM and 2.9 nM) for [^{3}H]paroxetine and [^{3}H]citalopram binding to rat cortical membranes respectively, being only 1.9- and 3.1-fold less potent than citalopram itself in the two binding assays. These results suggest that 5-IC has a high affinity for the 5-HT transporter sites and might be a potential SPET ligand. The binding of radio-iodinated 5-IC ([^{125}I]5-IC) to rat cortical membranes was inhibited by 5-IC (K_D = 2.9 nM), paroxetine (Ki = 0.12 nM), citalopram (Ki = 1.42 nM) and 5-HT (Ki = 248 nM). These Ki values corresponded well with data from [^{3}H]citalopram binding experiments and indicate that as for [^{3}H]citalopram, [^{125}I]5-IC is a suitable ligand for the 5-HT transporter.

Intravenous injection of [^{125}I]5-IC resulted in a rapid accumulation of radioactivity in rat brain. At 2hr after [^{125}I]5-IC injection, the highest level of specific [^{125}I]5-IC binding was observed in the brainstem (0.042 %D/g) and midbrain (0.037 %D/g), which are known to be rich in serotonergic neurons. Pretreatment with paroxetine caused a significant reduction in specific [^{125}I]5-IC binding in all brain

regions except cerebellum 2hr post-injection. The greatest reductions were observed in the midbrain (84.4%), rostral cortex (78.1%) and caudal cortex (63.2%). Smaller reductions occurred in the brainstem (49.8%) and hippocampus (34.8%). The reason for the brain regional variation in the inhibitory effect of paroxetine on specific [^{125}I]5-IC binding is not clear but these phenomena may indicate that a change in brain regional delivery of radioligand occurs by pretreatment with a pharmacologically active dose of drug, in this case paroxetine (1 mg/kg, i.v.). The results from the present work may indicate that [^{125}I]5-IC is a potential SPET ligand for the 5-HT transporter in human brain. Such a SPET ligand would be valuable for monitoring neuropsychiatric diseases such as depression and for detecting damage of serotonergic neurons by potent neurotoxins such as MDMA.

Binding of 5-HT$_{1A}$ receptor and 5-HT transporter ligands in rat cortex and hippocampus following cholinergic and serotonergic lesion

The cellular localisation of 5-HT$_{1A}$ binding sites and 5-HT uptake sites in the rat frontal cortex and hippocampus was examined using three different neurotoxins, ibotenate, AMPA and 5,7-DHT. Ibotenate and AMPA reduced ChAT activity in frontal cortex but not in hippocampus, indicating that both neurotoxins produced a selective destruction of cholinergic nerve terminals in frontal cortex. The lesions of basal forebrain cholinergic neurones using either ibotenate or AMPA did not effect the binding affinity (K_D value) and density (Bmax value) for

[^{3}H]8-OH-DPAT binding in either cortical or hippocampal membranes. These data suggest that the 5-HT$_{1A}$ binding sites are not localised on the cortical nerve terminals of cholinergic neurones situated in the basal forebrain. Therefore a loss of cortical 5-HT$_{1A}$ receptors in AD is unlikely to be due to a loss of cholinergic terminals. The destruction of serotonergic neurons by 5,7-DHT reduced markedly and significantly the 5-HT levels in rat frontal cortex and hippocampus in comparison with those in unlesioned animals, indicating a loss of serotonergic terminals. Lesioning of serotonergic neurones had no effect on [^{3}H]8-OH-DPAT binding in the cortex and hippocampus. Lesions of basal forebrain cholinergic neurones by ibotenate and AMPA did not effect the affinity or binding site density of [^{3}H]citalopram binding in either cortical or hippocampal membranes. These results suggest that the 5-HT uptake sites are not localised on cholinergic terminals in rat frontal cortex. Therefore a reduction of [^{3}H]citalopram binding sites observed in human frontal cortex with AD may not directly depend on damage of cholinergic terminals. Serotonergic lesions produced a reduction of binding site density for [^{3}H]citalopram binding in both the cortical and hippocampal membranes, suggesting that [^{3}H]citalopram can label the 5-HT transporter on serotonergic terminals in rat frontal cortex and hippocampus. The density of [^{3}H]citalopram binding sites was almost abolished in the hippocampus whereas its reduction in the frontal cortex was only 47.2%. The reason for the difference in the cortex and hippocampus remains unclear. This discrepancy may suggest that [^{3}H]citalopram labels a second population of binding sites in the frontal cortex that is not associated with serotonergic nerve

terminals. An alternative explanation is that the cellular distribution of the binding site in the cortex and hippocampus differs. A differential contribution of glial cells and neurones to the pattern of binding in the two brain areas could explain these data because the neurotoxic effect of 5,7-DHT would only be expected to destroy the neuronal [^{3}H]citalopram binding sites. In conclusion, the 5-HT$_{1A}$ receptors are not localised on cholinergic terminals in the cortex, or serotonergic terminals in the cortex and hippocampus. The 5-HT transporter sites are located on terminals of serotonergic neurons in the cortex and hippocampus, but not on cholinergic terminals in the cortex.

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APPENDIX

List of publications arising from this thesis

Permission from all authors concerned has been obtained.

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241P THE LOCALISATION OF 5-HT_{1A} AND 5-HT UPTAKE BINDING SITES IN THE RAT FOREBRAIN

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Although autoradiographic and binding studies have demonstrated the presence of 5-HT_{1A} sites in rat cortex and hippocampus, the ynaptic localization of these sites is not known (Pazos and Palacios, 1985). A loss of 5-HT_{1A} binding sites has, however, been)bserved post-mortem in frontal cortex obtained from Alzheimer's patients (Middlemiss et al, 1986). In the present study we have nvestigated whether 5-HT_{1A} and uptake binding sites are located on cholinergic and serotonergic nerve terminals in rat cortex and iippocampus.

Cholinergic neurones in the nucleus basalis were lesioned by direct injection of ibotenic acid (25 nmoles; AP -0.9; ML ±2.6; DV -).8mm). Rats were sacrificed after 7 days for the determination of choline acetyltransferase activity (ChAT, Fonnum, 1975) or for inding studies. Serotonergic neurones were destroyed by i.c.v. injection of 5,7-dihydroxytryptamine (150µg; animals pretreated for 0 min with 25mg/kg desmethylimipramine). Brain tissue was prepared for analysis of 5-HT levels or binding studies 14 days later. -HT_{1A} binding sites were labelled with [^{3}H]8-OHDPAT (0.5nM), using 10µM 5-HT to define specific binding and the 5-HT uptake ites using [^{3}H]citalopram (0.4nM), with 10µM citalopram used to define specific binding. Whole brain membranes were prepared rom frontal cortex and hippocampus of individual animals (n = 5 or 6 for each group).

'ollowing ibotenate injection ChAT activity was reduced by 49.44±2.43% (n = 6) in the frontal cortex but was unaltered 98.69±5.13%) in the hippocampus suggesting that the cholinergic innervation of the cortex had been selectively destroyed. No ignificant alteration in the number of binding sites was seen for [^{3}H]8-OHDPAT (Bmax [pmol/mg protein]; cortex, control = .45±0.02, lesion = 0.52±0.03; hippocampus, control = 1.83±0.06, lesion = 1.85±0.14) or [^{3}H]citalopram (Bmax [pmol/mg protein]; -ortex, control = 1.54±0.10, lesion = 1.33±0.11; hippocampus, control = 1.34±0.25, lesion = 1.37±0.31). Destruction of serotonergic ibres led to 77±2% and 78±5% reductions in cortical and hippocampal levels of 5-HT respectively. [^{3}H]Citalopram binding was educed by 45% in frontal cortex and was almost undetectable in the hippocampus of lesioned animals (Bmax [pmol/mg protein]; -ortex, control = 1.80±0.2, lesion = 1.00±0.16; $P < 0.05$). No alterations were observed in [^{3}H]8-OHDPAT binding (Bmax [pmol/mg rotein]; cortex, control = 0.68±0.04, lesion = 0.61±0.07; hippocampus, control = 1.96±0.12, lesion = 1.64±0.12). For both lesions iere was no significant alteration in the affinity of either ligand for its binding site.

'hese data suggest that the majority of [^{3}H]citalopram binding sites are located on the terminals of serotonergic neurones in rat cortex nd hippocampus. Binding of [^{3}H]8-OHDPAT was not altered following lesions of the cholinergic and serotonergic innervation of ie frontal cortex.

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258P COMPARISON OF AMPA AND IBOTENIC ACID LESIONS IN RAT CORTEX

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In our previous study employing ibotenic acid and 5,7-DHT lesions (Lawrence et al, 1991) , no evidence was found for a presynaptic localisation of $5HT_{1A}$ binding sites on cholinergic or serotonergic nerve terminals in the rat cortex or hippocampus.

This study has now been extended to include a unilateral excitotoxic lesion with the selective quisqualate receptor agonist, AMPA ((RS)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and to compare this lesion with that of ibotenic acid at the $5HT_{1A}$ site, labelled with [^{3}H]8-OHDPAT (0.5nM).

Cholinergic neurones in the substantia innominata were destroyed by direct injection of either ibotenic acid (25nmoles) or AMPA (7.5nmoles; AP - 0.9mm; ML ± 2.6mm; DV - 6.8mm). The lesions were verified by measurement of cortical and hippocampal levels of choline acetyltransferase (ChAT) after 7 days.

Table 1: ChAT activity and binding to $5HT_{1A}$ receptors following ibotenic acid and AMPA lesions

	Ibotenic acid (n = 6)		AMPA (n = 6)	
	control	lesion	control	lesion
CORTEX				
ChAT activity (nmoles/mg protein/60 min)	54.4 ± 3.9	27.3 ± 3.1	46.5 ± 4.1	15.1 ± 1.7
8-OHDPAT binding: K_D (nM)	1.38 ± 0.06	1.38 ± 0.06	1.21 ± 0.30	0.92 ± 0.21
B_{max} (pmol/mg protein)	0.45 ± 0.02	0.52 ± 0.03	0.49 ± 0.03	0.47 ± 0.05
HIPPOCAMPUS				
ChAT activity (nmoles/mg protein/60 min)	61.3 ± 5.8	62.3 ± 5.2	56.5 ± 5.5	55.9 ± 5.3
8-OHDPAT binding: K_D (nM)	1.51 ± 0.14	1.28 ± 0.08	1.11 ± 0.23	1.33 ± 0.11
B_{max} (pmol/mg protein)	1.83 ± 0.06	1.85 ± 0.14	1.61 ± 0.02	1.72 ± 0.03

Thus ibotenic acid and AMPA produced a similar level of neuronal destruction in the substantia innominata, as measured by the ChAT activity. However, for both lesions no changes were observed in either the density or affinity of $5HT_{1A}$ receptors in the cortex. This data confirms our previous observation that $5HT_{1A}$ receptors are not located on cholinergic terminals in the rat cortex.

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Binding of 5-HT_{1A} receptor and 5-HT transporter ligands in rat cortex and hippocampus following cholinergic and serotonergic lesions

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cellular localisation of 5-HT_{1A} receptor and 5-HT transporter binding sites in the rat cortex and hippocampus has been examined. Lesions of er basal forebrain neurones or serotonergic neurones did not affect [^{3}H]8-OH-DPAT binding, suggesting that 5-HT_{1A} binding sites are not lised on cholinergic or serotonergic nerve terminals. The binding of the 5-HT transporter ligand, [^{3}H]citalopram was unaffected by the linergic lesion whereas binding was reduced in both the hippocampus and cortex following serotonergic lesions. A reduction in binding site sity rather than an alteration in affinity was responsible for this effect. While these data suggest that [^{3}H]citalopram binding sites are located erotonergic nerve terminals, the abolition of hippocampal binding sites contrasted with a 50% loss in cortical tissue.

Recent advances in human brain imaging technology vide the basis for the development of diagnostic ts for pathological conditions such as Alzheimer's ease. Our group has concentrated on ligands for gle positron emission tomography (SPET) directed ards the 5-HT_{1A} subtype of serotonin receptors[10,11] is approach was prompted by the observation that HT_{1A} binding is reduced in the brain of Alzheimer's ients[2,4,14].

The present study used a brain lesioning approach study the cellular localisation of 5-HT_{1A} receptors in rat cortex and hippocampus. Binding to 5-HT_{1A} eptors was examined using [^{3}H]8-hydroxy-2-(di-n-pylamino)tetralin ([^{3}H]8-OH-DPAT) as the radiond[8,13,17,18]. A loss of both cholinergic and serotoner- markers has been reported in the brains of Alzmer's patients[2,16], and the reduction in 5-HT_{1A} ding in the cortex of these patients could be related the loss of either population of neurones. Excito- c lesions were used to mimic the loss of cholinergic ervation of the cortex observed in the brains of heimer's patients. In further experiments, lesions of serotonergic cell bodies that innervate the cortex and hippocampus were performed. Binding to the serotonin transport site using [^{3}H]citalopram was also studied in both control and lesioned animals since this has been shown to be a useful marker for serotonergic nerve terminals[5].

For excitotoxic lesions of basal forebrain cholinergic neurones, Cobb–Wistar rats weighing 280–300 g were anaesthetised with pentobarbitone (60 mg/kg) and placed in a Kopf stereotaxic frame. 0.5 μl of either ibotenate (Research Biochemicals Inc.; 25 nmol in 50 mM phosphate-buffered saline (PBS); pH 7.4) or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; Tocris Neuramin; 7.5 nmol in 50 mM PBS; pH 7.4) was injected into the basal forebrain (AP −0.9; ML ±2.6; DV −6.8) over a 5-min period. The needle was left in place for a further 5 min before removal. Animals were killed 7 days later either for binding studies or for determination of choline acetyltransferase (ChAT) activity[6]. Choline acetyltransferase (ChAT) activity in the frontal cortex ipsilateral to the excitotoxin injection site was 27.3 ± 3.1 ($n = 6$) and 15.1 ± 1.7 ($n = 6$) nmol/mg protein/h for ibotenate and AMPA lesions, respectively. Corresponding values

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the unlesioned contralateral cortex were 54.4 ± 3.9 = 6) and 46.5 ± 4.1 (n = 6) nmol/mg protein/h. The % reduction noted following AMPA injection was nificantly greater ($P < 0.01$) than the 50% reduction ed after ibotenate injection. In contrast, neither ion affected ChAT activity in the hippocampus (data shown).

Serotonergic cell bodies were destroyed using 5,7-dihydroxytryptamine (5,7-DHT; Sigma). Male Cobb–star rats weighing 280–300 g were anaesthetised h pentobarbitone (45 mg/kg, i.p.), and desmethnipramine (25 mg/kg, i.p.) was administered to prot noradrenergic neurones. After 30 min, 5,7-DHT 0 μg in 20 μl 0.9% saline containing 0.1% ascorbic d) was infused over 2 min into the lateral ventricle) −1.3; ML −1.7). The needle was left in place for rther 2 min before removal. After 14 days rats were d either for binding studies or neurochemical assment of the lesion by HPLC determination of 5-HT tent. Values for control (untreated) and lesioned ues are derived from separate groups of animals. IT levels in the cortex and hippocampus of 5,7-IT-lesioned animals were 1.23 ± 0.13 (n = 6) and 3 ± 0.09 (n = 6) pmol/mg wet weight, respectively. responding values in untreated animals were 5.53 1.43 (n = 6) and 4.1 ± 0.43 (n = 6) pmol/mg wet ght. Thus, the tissue content of serotonin was red by 78% and 83% in the cortex and hippocampus esioned animals.

For binding experiments, individual hippocampi and rtal cortices were homogenised in 40 vols. (v/w) cold 50 mM Tris-HCl (pH 7.4) and centrifuged at 00 × g for 10 min at 4°C. Membranes were washed mes followed by a 10-min incubation at 37°C to ove endogenous 5-HT. After a final centrifugation 0,000 × g, membranes were stored at −20°C. Imdiately before use, membrane suspensions were ved and diluted with 50 mM Tris-HCl (pH 7.4); ices 300 vols., hippocampi 500 vols. The protein tent of individual membrane suspensions was deterred according to the method of Bradford[3]. For the]8-OH-DPAT binding assay, aliquots of the memne suspensions were incubated with 0.5 nM [^{3}H]8--DPAT (264 Ci/mmol; Amersham International) 0 mM Tris-HCl (pH 7.4), containing 5 mM $MgSO_4$ ne absence or presence of 0.03–100 nM unlabelled H-DPAT. Incubations were terminated following a nin incubation at 25°C by rapid filtration through tman GF/B filters under vacuum. 10 μM 5-HT used to define non-specific binding. For the citalopram binding assay, samples of the memne suspensions were incubated in duplicate with 0.4 [^{3}H]citalopram (50 Ci/mmol; NEN) in 50 mM

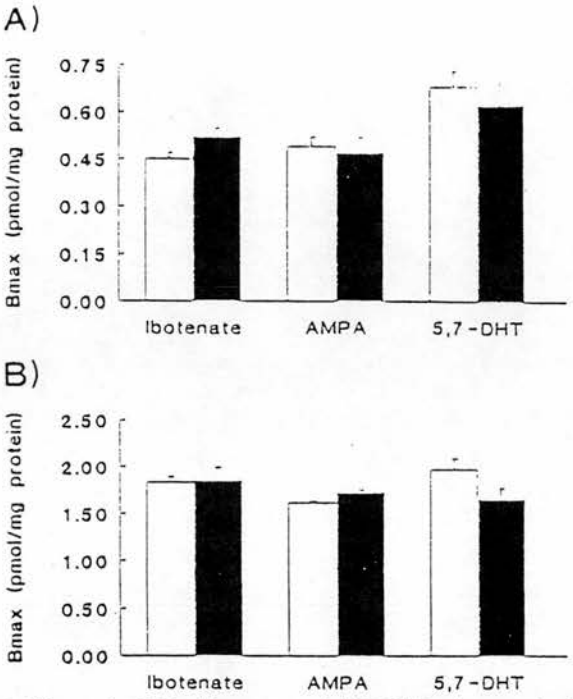
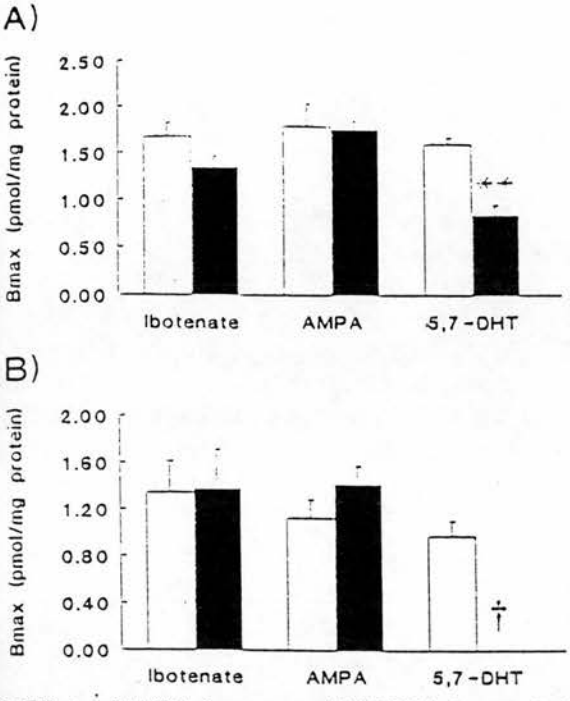


Fig. 1. Effects of AMPA, ibotenate and 5,7-DHT lesions on the B_{max} of [^{3}H]8-OH-DPAT binding to rat cortical (A) and hippocampal (B) membranes. 10 μM 5-HT was used to define non-specific binding. Data (pmol/mg protein) are the mean ± S.E.M. from 6 independent samples with control (open bars) and lesion (filled bars) data being derived from either the same rat (for ibotenate and AMPA lesions) or different animals (for 5,7-DHT lesions).

Tris-HCl, pH 7.4 containing 150 mM NaCl and 5 mM KCl in the absence or presence of 0.03–100 nM unlabelled citalopram. Incubations were carried out for 60 min at 25°C and terminated as described previously. Non-specific binding was defined using 10 μM citalopram. Binding data were fitted by least squares to the logistic expression $Y = MX^P/(X^P + IC_{50}^P)$, and K_d and B_{max} values were calculated.

In unlesioned tissue, the binding affinity (K_d) of [^{3}H]8-OH-DPAT in cortical and hippocampal membranes was 1.35 ± 0.06 nM and 1.45 ± 0.15 nM, respectively. In the case of [^{3}H]citalopram, the K_d in cortical and hippocampal membranes was 2.04 ± 0.26 nM and 2.25 ± 0.26 nM, respectively. The K_d of both [^{3}H]8-OH-DPAT and [^{3}H]citalopram for their respective binding sites on cortical and hippocampal membranes was not significantly affected by either excitotoxin or 5,7-DHT-induced lesions (data not shown).

Excitotoxic lesions of basal forebrain cholinergic neurones using either ibotenate or AMPA did not effect [^{3}H]8-OH-DPAT binding site density (B_{max}) in either cortical or hippocampal membranes (Fig. 1). The B_{max} was also unaffected in membranes prepared from the cortex and hippocampus of 5,7-DHT-lesioned animals (Fig 1). Basal forebrain cholinergic lesions using either ibotenate or AMPA did not affect the B_{max} of



2. Effects of AMPA, ibotenate and 5,7-DHT lesions on the B_{max} ^{3}H]citalopram binding to rat cortical (A) and hippocampal (B) nbranes. Non-specific binding was defined using 10 μM citalo-m. Data (pmol/mg protein) are the mean ± S.E.M. from 6 inde-dent samples with control (open bars) and lesion (filled bars) ı being derived either from the same rat (for ibotenate and PA lesions) or from different animals (for 5,7-DHT lesions). $P < 0.01$. †, specific binding of [^{3}H]citalopram in 5,7-DHT-le-ed hippocampal membranes was less than 10% of total binding. s precludes accurate determination of the B_{max} value by satura-tion analysis of the data.

H]citalopram binding to cortical or hippocampal mbranes (Fig 2). In contrast, 5,7-DHT lesions of otonergic neurones caused a marked reduction in B_{max} of [^{3}H]citalopram binding (Fig 2). The B_{max} :ortical membranes from lesioned animals was 47% < 0.01) of that in unlesioned animals. Although the ıx of [^{3}H]citalopram binding in hippocampal mem-nes from 5,7-DHT lesioned animals could not be urately determined (see Fig. 2 legend), specific ding at a ligand concentration of 0.4 nM was 0.04 ± l pmol/mg protein in lesioned hippocampi com-ed with 0.31 ± 0.02 pmol/mg protein in unlesioned pocampi. This suggests that the B_{max} was reduced greater than 85% in lesioned animals. The reduc-ı in specific [^{3}H]citalopram binding was significantly ater ($P < 0.05$) in hippocampal compared with cor-l membranes.

Data from the present study suggest that 5-HT_{1A} ding sites are not localised on the cortical nerve minals of cholinergic basal forebrain neurones. Both enate and AMPA lesions markedly reduced corti-ChAT activity without effecting [^{3}H]8-OH-DPAT ding to cortical membranes. The finding that AMPA lesions produced a larger reduction in cortical ChAT activity is consistent with recent reports[15]. These data should be contrasted with the results of release experiments which suggest that 5-HT_{1A} receptor agonists increase cortical acetylcholine release[1]. This discrepancy can be explained by an action of 5-HT_{1A} agonists, administered systemically in the release experiment, on cholinergic basal forebrain cell bodies resulting in altered cortical transmitter release. Serotonergic lesions did not affect [^{3}H]8-OH-DPAT binding in the cortex and hippocampus. While this finding is in agreement with previous data[8], a presynaptic localisation of 5-HT_{1A} binding sites has been demonstrated on serotonergic cell bodies in the raphe nucleus[18]. Although a non-significant 21% reduction in cortical [^{3}H]8-OH-DPAT binding following serotonergic lesioning has been reported[8], this finding was not confirmed in the present study.

The present study also examined the localisation of serotonin uptake sites using [^{3}H]citalopram. While cholinergic lesions were ineffective, serotonergic lesions reduced [^{3}H]citalopram binding in both cortical and hippocampal membranes. Although this confirms presynaptic localisation on serotonergic nerve terminals[5], there was a marked difference in the extent of the reduction in the cortex and hippocampus. Binding was virtually abolished in hippocampal membranes prepared from 5,7-DHT-lesioned rats, whereas a 50% reduction was noted in cortical membranes prepared from the same animals. Since serotonin levels were depleted to a similar extent in both brain areas, [^{3}H]citalopram may label a second population of binding sites in the cortex that is not associated with serotonergic nerve terminals. A possible explanation is that serotonin uptake sites exist in astrocyte cultures[9], and the cellular distribution (neuronal versus glial) of the binding site in the cortex and hippocampus may differ. However, serotonergic lesions using *p*-chloroamphetamine are reported to abolish [^{3}H]citalopram binding in both the hippocampus and cortex, and the degree of tissue serotonin depletion was similar to that observed in the present study[5]. Alternatively, the 5-HT system may be less uniform than at first thought[12], and a small component of the cortical system enriched in 5-HT uptake sites could either be less sensitive to the 5,7-DHT lesion or be protected from the lesion by desmethylimipramine pretreatment.

In conclusion, the majority of 5-HT_{1A} binding sites are not localised on cholinergic or serotonergic nerve terminals in the rat cortex and hippocampus. A postsynaptic localisation therefore appears likely and experimental evidence favouring this idea has been reported; [^{3}H]8-OH-DPAT binding is reduced both in hippocam-

and cortical pyramidal cells following kainate[8] and
xensin[7] lesions, respectively. If 5-HT_{1A} directed
gs can be developed as radioligands for PET and
ET studies, these agents are ideal for imaging corti-
cell loss rather than as presynaptic markers. We are
sently developing radioiodinated analogues of
lopram and paroxetine for the study of serotonergic
ve terminal loss in the cerebral cortex.

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Neurochemical and electrophysiological studies on FR115427, a novel non-competitive NMDA receptor antagonist

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`he pharmacological profile of FR115427 has been examined using ligand binding and electrophysiological techniques. ding of [^{3}H]dizocilpine in the presence of L-glutamate was inhibited by the (+) isomers of dizocilpine and FR115427. The esponding (−) isomers were less active, and stereoselectivity was particularly marked in the case of FR115427. In contrast to cilpine, the affinity of FR115427 for [^{3}H]dizocilpine binding sites was little affected by addition of either L-glutamate and/or ine. In a cortical wedge preparation, FR115427 inhibited N-methyl-D-aspartate (NMDA)-induced responses in a non-compe-e, use-dependent manner. Intracellularly recorded excitatory synaptic responses in hippocampal neurones were only partially bited by FR115427 thereby confirming a selective effect on the NMDA-mediated component of neuronal excitation induced he endogenous neurotransmitter. The data suggest that FR115427 is a non-competitive, use-dependent NMDA receptor gonist with more pronounced stereoselectivity and less marked use dependence than dizocilpine.

NMDA (N-methyl-D-aspartate); Non-competitive antagonism; Dizocilpine; FR115427; Cortical wedge; Hippocampus

ntroduction

`he N-methyl-D-aspartate (NMDA) receptor pro- has recently been cloned (Moriyoshi et al., 1991). ırochemical and electrophysiological studies have ıonstrated that a number of pharmacologically dis- t modulatory sites are present within its structure recent reviews by Reynolds and Miller, 1990; ng and Kemp, 1991). Research efforts aimed at eloping compounds that selectively interact with e sites are ultimately directed at regulating NMDA ptor function in various disease states. The NMDA ptor subtype has been implicated in a range of nal and pathological conditions including synaptic ticity and learning (Lester et al., 1988), and neu- ıthological cell death (Meldrum and Garthwaite,); Olney, 1990). NMDA receptor activation can be bited in a competitive manner by antagonists acting ıe site that recognises the endogenous neurotransmitter, and a number of pharmacological agents have been developed that selectively interact with this site (see Olverman and Watkins, 1989). Receptor function can also be blocked in a non-competitive manner by drugs such as (+)-5-methyl-10,11-dihydro-5H-dibenzo-[a,d]cyclohepten-5,10-imine maleate (dizocilpine or MK801) and phencyclidine (PCP) that interact with sites located within the NMDA receptor-associated ion channel (Wong et al., 1986, 1988; MacDonald and Nowak, 1990; MacDonald et al., 1990; Lodge and Johnson, 1990).

In this study we have examined the inhibitory effect of the novel isoquinoline derivative, (+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline hydrochloride (FR115427; fig. 1) on [^{3}H]dizocilpine binding to rat brain membranes. Both the stereoselectivity of this interaction and the effects of L-glutamate and glycine on the affinity of FR115427 for [^{3}H]dizocilpine binding sites have been studied. The functional significance of this interaction has been studied using a cortical wedge preparation to determine the mode of action of FR115427 and, in particular, to examine whether the observed inhibition of NMDA-mediated responses is competitive or non-competitive with use-dependent properties. The effects of FR115427 on the excitatory

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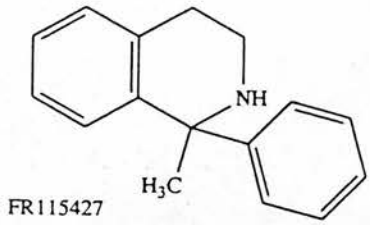
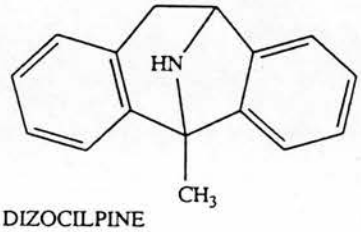


FR115427



DIZOCILPINE

1. Chemical structures of dizocilpine and FR115427 ((+)-1-methyl-1-phenyl-1,2,3,4 tetrahydroisoquinoline HCl).

tsynaptic potential (e.p.s.p.) elicited in hippocampal l neurones by stimulation of Schaffer collateral-ımisural fibres in the stratum radiatum have also n examined in order to examine the action of 115427 on responses produced by the endogenous tatory amino acid transmitter.

Materials and methods

Preparation of rat brain membranes

he cerebral cortices of male Cob-Wistar rats (250–g) were dissected and homogenised in 15 volumes w) of ice-cold 0.32 M sucrose using a glass teflon ıogeniser. The homogenate was centrifuged at 1000 at 4°C for 10 min, and the supernatant recen-ıged (17000 × *g*, 20 min, 4°C). The resultant P_2 et was lysed with 30 volumes of glass distilled ɜr. After incubation at 37°C for 30 min, the mem-ıe suspension was centrifuged at 50000 × *g* for 10 at 4°C, the supernatant discarded and the pellet hed by resuspension in glass distilled water (30 mes) and centrifugation (50000 × *g*, 4°C, 10 min). resultant pellet was resuspended to 10 volumes of s distilled water and stored at −20°C. The mem-ıe suspension was thawed prior to use and diluted 0 volumes with glass distilled water, recentrifuged)00 × *g*, 4°C, 10 min) and the final pellet was resus-ded in 30 volumes of 5 mM Tris-HCl (pH 7.4, 20°C) kept on ice until required. The protein content of membrane suspension was determined by the ıod of Bradford (1976), using bovine serine albu-(fraction V) as the standard.

2.2. [^{3}H]Dizocilpine binding assay

[^{3}H]Dizocilpine (24 Ci/mmol; NEN; 1 nM final ligand concentration) was preincubated at 25°C with 5 mM Tris-HCl buffer (pH 7.4), and increasing concentrations of test drugs ((+)-FR115427, 1 nM–30 μM; (−)-FR115427, 3 nM–100 μM; (+)-dizocilpine, 0.1 nM–3 μM; (−)-dizocilpine, 1 nM–30 μM) for 2 min prior to addition of 0.5 ml of the membrane suspension (300–500 μg protein) to give an assay volume of 1 ml. The mixture was vortexed briefly then incubated for 120 min at 25°C. L-Glutamate and glycine, when present, were at a final concentration of 10 μM. Non-specific binding was determined in the presence of 30 μM dizocilpine. Incubations were terminated by rapid filtration through Whatman GF/B filters using a Brandel cell harvester, followed by two washes with 5 ml of 5 mM Tris-HCl buffer. Filters were transferred to scintillation vials and 100 μl of 100% formic acid was added to digest membrane protein followed 10 min later by 4 ml of Emulsifier Safe Scintillant. Radioactivity was measured in a Canberra-Packard 1900CA liquid scintillation analyser using automatic quench correction.

2.3. Cortical-wedge experiments

Cortical slices (500 μm thick) were prepared from the brains of male Cobb-Wistar rats (200–250 g). The brain was quickly removed and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF) of the following composition (in mM; sodium chloride 134; potassium chloride 2.75; sodium hydrogen carbonate 16; calcium chloride 2.5; magnesium sulphate 2; potassium dihydrogen phosphate 1.25; D-glucose 10). A 3–4 mm slice of the ventral surface of the brain was removed and discarded. The remaining tissue was cut transversely just anterior to the cerebellum and at the level of the optic chiasm. The tissue between these cuts was further trimmed laterally to give a block about 8 mm square. This was fixed with cyanoacrylate glue to the mounting block of the vibratome (Vibroslice, Campden Instruments) with the ventral aspect opposing the agar block used for support. The slices were further trimmed to the shape of small wedges about 1 mm wide at the cortical surface. They were maintained at the gas-liquid interface of a stationary interface chamber in an ACSF containing magnesium with a gas phase of 95% oxygen and 5% carbon dioxide. After 60 min incubation at room temperature one slice was transferred to the two-chambered bath and both chambers were perfused independently at 2 ml/min with oxygenated magnesium free ACSF. The design of the bath was essentially as described previously (Harrison and Simmonds, 1985). The wedge-shaped slices were positioned so they passed through the grease filled slot with a large proportion of

cortex in one compartment and all of the corpus losum and a small area of ventral cortex in the other mpartment. The potential difference between the compartments was measured with silver/silver oride electrodes in agar connected to a dc amplifier rass model 7P1/7DA), displayed on a chart recorder ould model 2200S) and also relayed to a modified ital audio processor (Sony model PCM 701ES) and red on videotape. The relationship between NMDA ncentration and depolarisation was determined be- e exposure of the slice to FR115427. Depending on requirements of the particular experiment, 115427 was left in contact with the preparation for -20 min before responses to appropriate concentra- ns of NMDA were determined. Four point Schild ts were constructed using doses of FR115427 of 5–5 μM. In some experiments tetrodotoxin (0.1 M) was added to the ACSF to inhibit spontaneous leptiform spiking; this did not affect the depolarisa- n produced by NMDA (Harrison and Simmonds, 5). Schild plots were generated from multiple dose- ponse relationships using different concentrations of 115427 by plotting the logarithm of the concentra- n of FR115427 against the logarithm of (DR − 1), ere DR (dose ratio) = D_a/D. D_a is the dose of MDA needed to achieve a depolarisation of a given in the presence of the antagonist and D is the dose NMDA which produces an identical response in the ence of the antagonist. In order to obtain a four- nt Schild plot a preparation had to be viable and le for at least 10 h; some of the preparations in this dy still gave consistent responses 14 h after setting (R,S)α-Amino-3-hydroxy-5-methyl-4-isoxazole- prionic acid (AMPA), a non-NMDA receptor ago- was applied at the end of each NMDA dose-re- nse relationship to ensure that there was no signifi- t deterioration in the integrity of the preparation. data reported here were obtained from 21 cortical es.

2.4. *Intracellular recording from hippocampal CA1 neurones*

Transverse slices of rat hippocampus, cut using a vibratome at a thickness of 400 μm, were placed on nylon netting in an interface chamber at 35°C. They were left to equilibrate for about 60 min before recordings were made from pyramidal neurones in the CA1 region. A pair of fine tungsten wire stimulating electrodes insulated except at the tip were placed on the stratum radiatum and were connected to a Grass S88 stimulator via a photoelectric stimulus isolation unit (PSIU6, Grass Instruments). Intracellular recordings were obtained with microelectrodes made from thick walled (1.2 mm o.d.) glass capillary tubing (GC 120F, Clark Electromedical Instruments) pulled on a conventional puller (Flaming Brown P80/PC). When filled with 2 M potassium acetate the electrodes had DC resistances of 70–100 MΩ. Signals were recorded with an Axoclamp 2A amplifier (Axon Instruments) displayed on a Gould chart recorder, and were relayed to a modified digital audio processor and stored on videotape for later analysis. E.p.s.p.'s were evoked with stimulating currents of 10–20 μA; the e.p.s.p. often appeared to consist of two components in low magnesium solutions containing picrotoxin (100 μM).

2.5. *Materials*

The (+) and (−) isomers of both FR115427 and dizocilpine were synthesised in the New Drug Research Laboratories, Fujisawa Pharmaceutical Co. Ltd., Osaka, Japan. AMPA was obtained from Tocris Neuramin, Bristol, U.K. All drugs were dissolved in deionised water or equivalent NaOH prior to experimentation. Other drugs and reagents were obtained from Sigma and were of the highest available purity.

BLE 1

alues for stereoisomers of dizocilpine and FR115427 in the presence and absence of L-glutamate (10 μM) and/or glycine (10 μM).

affinity of the (+) and (−) isomers of dizocilpine and FR115427 for [^{3}H]dizocilpine binding sites on rat cortical membranes was studied in presence or absence of 10 μM L-glutamate, glycine or a combination of these amino acids in the assay buffer. Membranes were incubated [^{3}H]dizocilpine (1 nM) in absence or presence of increasing concentrations of test drug for 120 min at 25°C. Bound and free ligand were rated using a Brandell Cell Harvester. K_i values were calculated from the equation $K_i = IC_{50}/(1+L/K_D)$. Data represent mean es ± S.E.M. of the number of separate determinations indicated in parentheses.

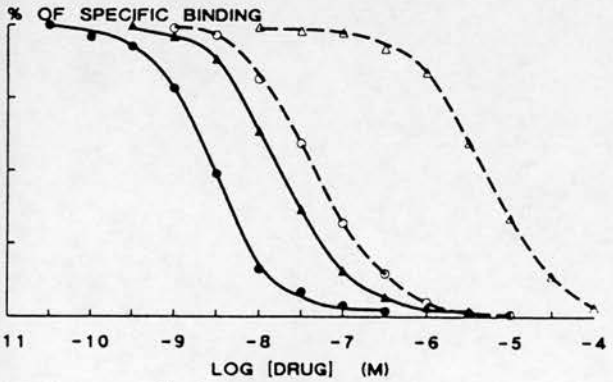
g	K_i (nM)			
	No addition	L-Glutamate	Glycine	Combination
Dizocilpine	19.4 ± 4.2 (9)	3.14 ± 0.3 (9)	5.7 ± 0.5 (4)	2.9 ± 0.4 (4)
Dizocilpine	30.6 ± 3.4 (7)	15.1 ± 1.1 (7)	N.D.	N.D.
FR115427	76.8 ± 17.7 (7)	43.3 ± 4.0 (7)	45.9 ± 3.1 (4)	45.3 ± 6.2 (4)
FR115427	4516 ± 887 (7)	4369 ± 526 (7)	3916 ± 374 (4)	4570 ± 699 (4)

Results

[^{3}H]Dizocilpine binding experiments

FR115427 inhibited [^{3}H]dizocilpine binding with a value of 43.3 nM (fig. 2 and table 1) in the presence 10 μM L-glutamate. Dizocilpine was found to be fold more potent as an inhibitor of binding with a value of 3.14 nM under identical conditions (fig. 2 table 1). The difference in affinity was less appar- in the absence of added L-glutamate; K_i values of 4 and 76.8 nM were obtained for dizocilpine and 115427 respectively demonstrating a 4-fold differ- e in affinity (table 1). Inhibition of binding by both npounds exhibited stereoselectivity but this was far e pronounced in the case of FR115427. When npared with the corresponding (+) isomer, the (−) ners of FR115427 and dizocilpine were respectively - and 4.8-fold less potent as inhibitors of [^{3}H]di- ilpine binding in the presence of 10 μM L-gluta- e (fig. 2 and table 1). K_D and B_{max} values for]dizocilpine binding were determined in the pres- e of various fixed concentrations of FR115427 (25– nM). Increasing concentrations of FR115427 re- ed the binding affinity of [^{3}H]dizocilpine but the ding site density was unaltered indicating that 115427 was a competitive inhibitor of binding (data shown). The competitive nature of the interaction R115427 and [^{3}H]dizocilpine for the binding sites confirmed by Dixon analysis which gave a K_i value FR115427 of 32 nM. This suggests that the two gs interact with a common binding site.

he binding of [^{3}H]dizocilpine was modulated by presence of L-glutamate and/or glycine in the assay buffer (table 1). The affinity of (+)-dizocilpine was increased by 6.2-fold from 19.4 to 3.14 nM in the presence of 10 μM L-glutamate. Maximal stimulation of binding was noted at this concentration of L-glutamate, and the effect involved only an alteration in the affinity of the binding site for dizocilpine rather than an increase in the number of binding sites (unpublished data). The affinity of (−)-dizocilpine was increased 2-fold under identical conditions (table 1). In contrast, the presence of L-glutamate in the assay buffer had little effect on the affinity of either (+)- or (−)-FR115427 for [^{3}H]dizocilpine binding sites (table 1). The affinity of (+)-dizocilpine was also increased by 3.4-fold when 10 μM glycine was included in the assay buffer (table 1). An effect of 10 μM glycine on the affinity of (+)- and (−)-FR115427 was almost undetectable (table 1). In the presence of a combination of 10 μM L-glutamate and 10 μM glycine the affinity of (+)-dizocilpine for [^{3}H]dizocilpine binding sites was increased by 6.7-fold (table 1), an identical increase to that observed in the presence of L-glutamate alone (i.e. there was no indication of an additive effect). Little change in the affinity of (+)- and (−)-FR115427 was noted in the presence of 10 μM L-glutamate and 10 μM glycine (table 1).

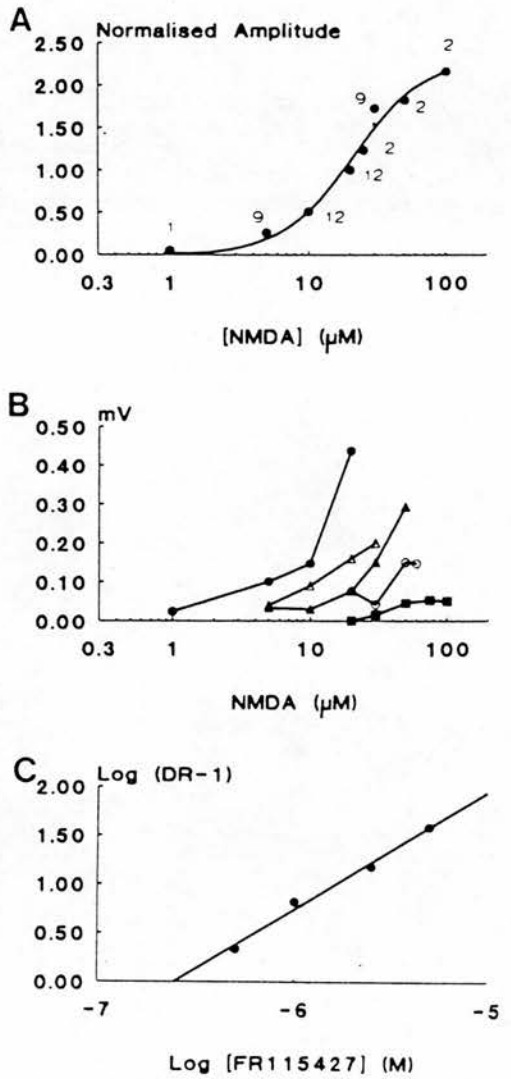


2. Inhibition of [^{3}H]dizocilpine binding by the (+) and (−) rs of dizocilpine and FR115427. Cortical membranes were ated with [^{3}H]dizocilpine (1 nM) for 120 min in the presence or ce of varying concentrations of test compound. All assays were rmed in the presence of 10 μM L-glutamate. Bound and free l were separated using a Brandell Cell Harvester. Data are a representative experiment. Each point is the mean value of ate determinations. (+)-Dizocilpine (filled circles); (−)-di- ine (filled triangles); (+)-FR115427 (open circles); (−)-FR115427 (open triangles).

3.2. Cortical wedge experiments

In the majority of preparations exposure to magnesium-free ACSF led to the appearance of spontaneous epileptiform spikes. The depolarisation induced by NMDA was dose-dependent. The maximal response was obtained at 100 μM, and the relationship was sigmoidal with an EC_{50} of 21 μM (fig. 3A). Repetitive challenges with the same dose gave consistent responses which did not desensitise (fig. 4A, C). Exposure of the preparation to FR115427 (1 μM) had three predominant actions: (i) a gradual reduction in the amplitude of the depolarisation produced by NMDA (fig. 4B, D), (ii) a reduction in the frequency of the associated epileptiform-spike activity (fig. 4B), and (iii) a reduction in the amplitude of the epileptiform spikes (fig. 4B). The antagonism appeared to be selective for the NMDA receptor since responses to the non-NMDA agonist AMPA were relatively unaffected by FR115427 (1 μM), a concentration that reduced the response to NMDA by up to 50% (fig. 5C, D). Increasing the dose of NMDA overcame the antagonism, but only transiently, since a second challenge with the same dose of NMDA resulted in a smaller depolarisation (data not shown). The Schild plot generated from the data in fig. 3B had a slope of 1.21 whereas a slope of 1 would be expected for a competitive antagonist. Extrapolation of the line gave a pA_2 of 6.6 (fig. 3C). The corresponding data from a second preparation gave a slope of 1.6 and a pA_2 of 6.45.

Additional experiments investigated the possible use pendence of FR115427. The response of cortical dge preparations to NMDA was first examined at a ed time after exposure to FR115427 (fig. 5A, B). The



3. (A) Composite dose-response relationship showing the nor-sed DC shift vs. NMDA concentration. The data were obtained i 12 different preparations with the response to 20 μM NMDA ı the value 1.0. The numbers beside each point denotes the ber of preparations of the 12 that received that dose. The line fitted to the data points using the logistic equation ($Y = {}^{P}/(X^{P}+K^{P})$ where M is the maximum response, K is the dose ucing half the maximum response and P determines the steep- of the curve. (B) The dose-response relationships for NMDA shifted to the right with increases in the concentration of 15427 (0.5–5 μM). The filled circles show the control dose-re-se relationship. The rightward shift was accompanied by a fall in naximal response. FR115427 (1 μM) caused an 83% fall in the ıitude of the depolarisation elicited by NMDA (20 μM) and this entration of FR115427 was chosen for the grease-gap experi- s reported here. The doses of FR115427 used were 0.5 μM ı triangles), 1.0 μM (closed triangles), 2.5 μM (open circles) and M (filled squares). (C) A Schild plot made from these data gave a pA_2 value of 6.6, the slope of the line was 1.2.

size of the depolarisation relative to control was then compared with that recorded in a second experiment in which an identical preparation was exposed to NMDA on several occasions following addition of FR115427. The corresponding test NMDA response was then measured at the same time interval used in the first experiment (fig. 5C, D). Use dependence would be manifest if the response relative to control in the second experiment were smaller than the first; in the absence of use dependence both responses would be identical. A significant difference was found between the two values in the present study (fig. 5E). The depolarisation produced by a single challenge of NMDA after exposure to FR115427 for 70 to 108 min was on average 83 ± 20% (n = 5) of the control value. In contrast, the size of the depolarisation produced by NMDA following exposure to the antagonist (84–107 min exposure) in preparations challenged several times in the intervening period with NMDA was 48.5 ± 15% (n = 4) of control. The difference between these values was significant ($P < 0.03$, two-tailed t-test; fig. 5E). In some preparations, there was also a small fall in the amplitude of the AMPA depolarisations. The control amplitude of AMPA responses was 0.6 ± 0.13 mV (n = 8) which fell to 0.57 ± 0.14 mV (n = 8) in the presence of FR115427 (1 μM). This decrease possibly reflects a slight deterioration in the preparations following multiple challenges with NMDA. The decrease was not significant ($P > 0.07$, two-tailed t-test).

3.3. Intracellular recording

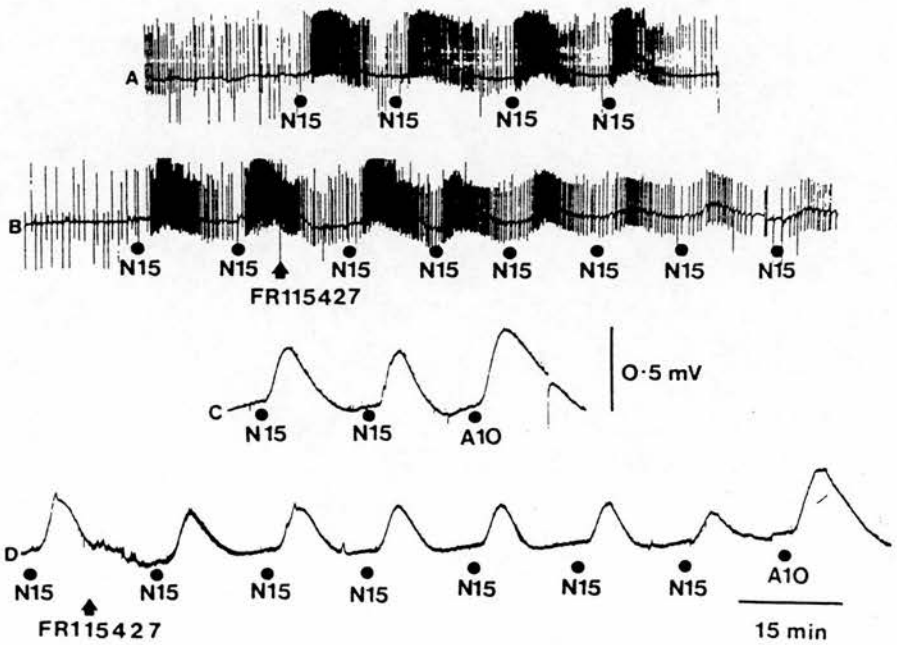
Hippocampal CA1 pyramidal neurones became hyperexcitable and discharged bursts of action potentials in bathing solutions containing no added magnesium and 100 μM picrotoxin to block γ-aminobutyric $acid_A$ ($GABA_A$)-mediated inhibition. Stimulation of the stratum radiatum elicited an e.p.s.p. accompanied by a discharge of action potentials which made it impossible to accurately measure the amplitude of the underlying synaptic potential. In three preparations it was possible to elicit a subthreshold e.p.s.p. that was either a monophasic (fig. 6A) or a dual component depolarisation (fig. 6B). Exposure to FR115427 (15 μM) reduced the amplitude of the second component in the latter category with little action on the first component. The action of FR115427 on the monophasic e.p.s.p. was only detectable when the control and test e.p.s.p. were superimposed since the decay phase of the e.p.s.p. was reduced whereas the peak amplitude was not affected (fig. 6C). The mean amplitude of the second component, or in the case of the monophasic e.p.s.p. the amplitude of the decay phase at 170 ms, was significantly reduced from a mean of 11.8 ± 3.2 to 8.1 ± 4.2 mV ($P = 0.037$; paired t-test; n = 3). The spontaneous epileptiform spiking observed in low magnesium solu-

ns containing picrotoxin was blocked by prolonged posure to FR115427 (15 μM).

Discussion

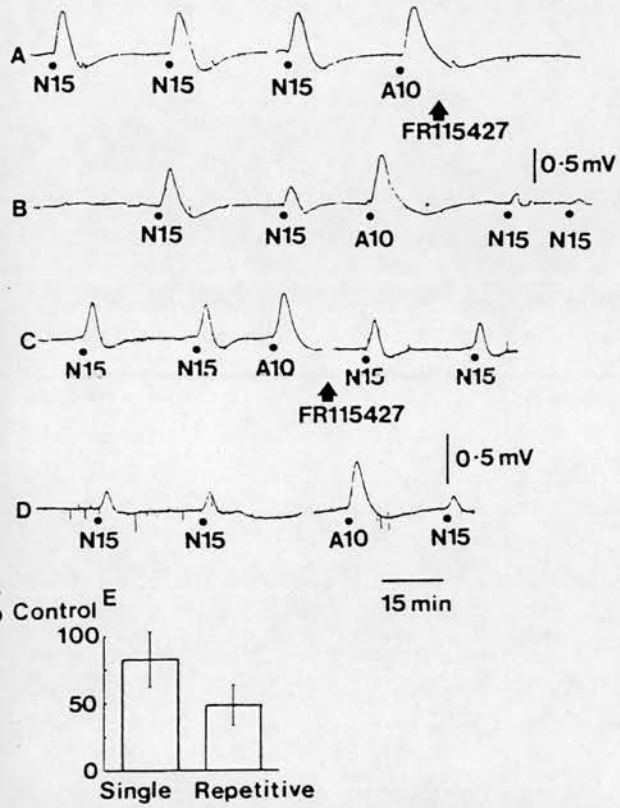
Radioligand binding studies using [^{3}H]dizocilpine arly show that the novel isoquinoline derivative 115427 inhibits binding of this ligand to rat brain mbranes. While FR115427 is 14-fold less potent n dizocilpine, the more pronounced stereoselectivity ibited by FR115427 compared with dizocilpine rep- ents a major difference between the two com- unds. This difference is indeed marked with the K_i os for the (+) and (−) isomers of FR115427 and ocilpine being 100- and 4.8-fold respectively in our of the more active (+) isomers. Further differ- es were revealed when the effects of L-glutamate l glycine on the affinity of FR115427 and dizocilpine [^{3}H]dizocilpine binding sites were examined. In the e of dizocilpine, the affinity of the (+) isomer was eased by 6.2- and 3.4-fold following addition to the ay buffer of L-glutamate and glycine respectively. ding of either glutamate or glycine to their recogni- n sites on the NMDA receptor presumably causes a conformational change that facilitates the interaction of dizocilpine with its binding site within the NMDA receptor associated ion channel (Foster and Wong, 1987; Reynolds and Miller, 1988). It was therefore of interest to note that the affinity of FR115427 for [^{3}H]dizocilpine binding sites was little affected by either L-glutamate or glycine. While the precise significance of this finding remains unclear, it may be interpreted as evidence that dizocilpine and FR115427 do not interact with an identical binding site. However, detailed analysis of the interaction of FR115427 with the binding site for [^{3}H]dizocilpine provided direct evidence that the same site is involved. In the presence of increasing concentrations of FR115427, the density of [^{3}H]dizocilpine binding sites was not altered whereas binding site affinity was reduced. Dixon analysis of these data confirmed that FR115427 competitively inhibited [^{3}H]dizocilpine binding. The access of FR115427 to the same binding site may therefore be less dependent on the ongoing activity of the NMDA receptor.

Electrophysiological experiments using a cortical wedge preparation demonstrate that blockade of NMDA-mediated responses by FR115427 is non-competitive. The evidence for this is (1) the slopes of the



4. Inhibition of NMDA depolarisation and spontaneous epileptiform-like activity in a rat cortical slice preparation by FR115427. (A) tion of 15 μM NMDA (N15) to the solution entering the chamber containing the grey matter is indicated by solid circles. This resulted in a otential change (depolarisation of the preparation is indicated by an upward shift) and a pronounced increase in the discharge frequency of pileptiform spikes; the response was repeatable and consistent. (B) FR115427 (1 μM) was added (solid arrow) to the solution perfusing the natter. Repeated challenges with NMDA resulted in a significant fall in the amplitude of the NMDA depolarisations and a reduction in the ency of the associated epileptiform spikes. Note that the frequency and amplitude of the spontaneous epileptiform spikes was also reduced. pontaneous epileptiform activity was almost completely abolished in another preparation exposed to tetrodotoxin (0.1 μM) but the arisations produced by 15 μM NMDA (N15) and 10 μM AMPA (A 10) were still seen. (D) After exposure to FR115427 (1 μM; solid), the NMDA depolarisations were significantly reduced in amplitude and fell by 40% relative to control whereas the AMPA depolarisations fell by only 15% relative to control.

ild plots were greater than 1, (2) antagonism of IDA-induced depolarisation was use-dependent and ld not be overcome, except transiently, by increas-



5. Use-dependent inhibition of NMDA-induced depolarisation R115427. (A) Depolarisations produced by 15 μM NMDA (N15) 10 μM AMPA (A10) in a rat cortical wedge preparation. etitive challenges with NMDA gave consistent responses in con- nagnesium-free ACSF (with 0.1 μM tetrodotoxin added). At the w FR115427 (1 μM) was added to the ACSF perfusing the grey er and left in contact with the preparation throughout the riment. (B) When 15 μM NMDA was added 81 min after sure to FR115427 (first response) the NMDA depolarisation not significantly altered although subsequent challenges with 15 NMDA produced smaller depolarisations. The response to 10 AMPA was unchanged. The break in record (A) represents 18 and the interval between records (A) and (B) is 16 min. (C) olarisations produced by 15 μM NMDA (N15) and 10 μM A (A10) in another cortical wedge preparation exposed to dotoxin. At the arrow FR115427 (1 μM) was added to the F perfusing the grey matter and was left in contact with the aration throughout the experiment. (D) The NMDA depolarisa- recorded 81 min after exposure to the antagonist (first response) own in this record. It was significantly smaller than the control nse in (C) although the response to 10 μM AMPA was not icantly different. The break in (C) represents 20 min and that in 5 min, the records at the end of (C) and the beginning of (D) ontinuous. (E) Histograms showing the amplitude of NMDA larisations as a percentage of control after exposure to FR115427 M) for periods of 70–108 min (single) and 84–107 min (repe-). The data in the column labelled single were obtained from trations unchallenged by NMDA prior to application of the test of NMDA whereas the data in the column labelled repetitive obtained from preparations challenged several times with A prior application of the test dose. A significant difference found between the two groups, the error bars denote the S.D.

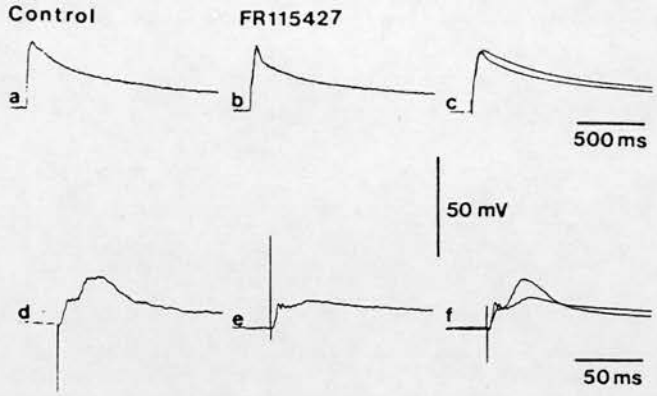


Fig. 6. The effect of FR115427 (15 μM) on the e.p.s.p. recorded in hippocampal CA1 pyramidal neurones in response to stimulation of the stratum radiatum. The preparations were exposed to magnesium-free ACSF containing picrotoxin (100 μM). The records are averages of eight successive sweeps. (a) In this neurone a monophasic e.p.s.p. was recorded, $E_m = -59$ mV. Exposure to FR115427 led to a more rapid decay of the falling phase of the e.p.s.p. (b). Superimposition of the records in (a) and (b) shows that the peak amplitude of the e.p.s.p. was relatively unchanged whereas a clear effect can be seen on the falling phase of the e.p.s.p. (c). (d) In another neurone an e.p.s.p. consisting of two components was recorded, $E_m = -63$ mV. FR115427 (15 μM) reduced the second component of the e.p.s.p. (e). Superimposition of the records in (d) and (e) shows a dramatic reduction in the amplitude of the second component whereas the first component was relatively unaffected (f).

ing the dose of NMDA, (3) dose-response plots for NMDA were progressively shifted to the right by increasing doses of FR115427, but the shift was not parallel and was accompanied by a corresponding fall in the maximal response to NMDA. The values obtained for pA_2 from the Schild plots derived from two experiments were 6.45 and 6.6. However, it is difficult to ascribe a significance to these values since the slopes of the Schild plots were greater than unity. Simmonds (1990) does make the point that having established that an antagonist is non-competitive it is still useful to construct a Schild plot to determine the concentration-effect characteristics of compounds within a group of non-competitive antagonists. In this regard, it is of interest that a pA_2 of 5 was obtained for ketamine (Harrison and Simmonds, 1985), which also acts non-competitively by blocking the ion channel of the NMDA receptor (MacDonald et al., 1987). We have also attempted to determine a pA_2 value for dizocilpine but this proved impossible because of the extreme use dependence of this drug. However, in the present study 2.5–5.0 μM of FR115427 was required to completely block responses to NMDA whereas under similar conditions 0.35 μM dizocilpine was required to achieve the same result suggesting that dizocilpine is an order of magnitude more potent than FR115427.

When the cortical wedge preparation was exposed to FR115427, a small component (on average 17%) was

nd to be use-independent. This was the proportion the NMDA response that was blocked in the ab- ce of prior exposure to NMDA. It is possible that slowly developing depolarisation induced by NMDA lects use dependence during the rising phase of the ial response leading to depression of its amplitude lliwell et al., 1989). Although the experiments to ablish use-dependence were carried out in the pres- e of tetrodotoxin in order to block spike-dependent ivity, spontaneous release of transmitter may still e occurred allowing FR115427 access to its binding . Alternatively, FR115427 may gain access to its ding site other than through the ion channel of the eptor. The latter possibility could be related to the erence in the extent of use-dependent blockade of MDA-mediated responses by FR115427 and di- ilpine noted in the present study.

After a period of equilibration in magnesium-free, odotoxin-free solutions the cortical wedge exhibited ntaneous epileptiform discharges that consisted of ltiple rapid spike-like depolarisations superimposed a depolarisation with a much slower time course. frequency of these depolarisations was increased NMDA, whereas the frequency and amplitude of h were reduced by FR115427. These observations gest that NMDA plays a role in the generation of n potentials and that this response is blocked by 115427 in parallel with the inhibition of NMDA-in- ed depolarisations. Similar results have been re- ted for dizocilpine (Wong et al., 1986).

he intracellular study on hippocampal CA1 neu- es in a brain slice preparation demonstrated that 115427 selectively blocked a second delayed compo- t of the e.p.s.p. evoked in magnesium-free, picro- n containing ACSF. This response is reported to be liated by NMDA receptors (Collingridge et al., 8; Hestrin et al., 1990). This observation compli- its data obtained with the cortical wedge prepara- thereby confirming the NMDA receptor as the is of action of FR115427. The initial component of e.p.s.p. that is mediated by non-NMDA receptors llingridge et al., 1988) was little effected by 15427. This finding is in keeping with cortical ge experiments in which FR115427 did not affect PA-induced depolarisation.

he binding experiments described in this paper gest that FR115427 and dizocilpine interact with a lar site on the NMDA receptor, and functional ies using the cortical wedge and hippocampal slice parations confirm this pharmacological profile. In- tion of NMDA-mediated responses by FR115427 sent data) and dizocilpine (Kemp et al., 1987) lved a non-competitive and use dependent mecha- n of action. These functional properties are charac- tic of non-competitive antagonists that interact with te(s) within the NMDA receptor-associated ion channel (MacDonald and Nowak, 1990; Lodge and Johnson, 1990). Data obtained in the hippocampal slice preparation demonstrate that FR115427 inhibits the NMDA-mediated component of neuronal excitation evoked by endogenous transmitter in a specific hippocampal pathway.

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Characterisation of the binding of [^{3}H]FR115427, a novel non-competitive NMDA receptor antagonist, to rat brain membranes

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he binding of [^{3}H]FR115427 ([^{3}H](+)-1-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline) to rat cortical synaptosomal mem-ıes was investigated. Binding was optimal at pH 7.4–8.0, and temperature had little effect on specific binding. Binding hed equilibrium within 30 min at 25°C, and was reversible in the presence of excess unlabelled FR115427. [^{3}H]FR115427 nd to a single population of non-interacting sites with an affinity of 45.4 ± 3.9 nM, and a binding site density of 9.12 ± 0.52 ıl/mg protein. The affinities of other *N*-methyl-D-aspartate (NMDA) receptor channel blockers for [^{3}H]FR115427 binding ; were consistent with binding to a similar site to that occupied by dizocilpine. Binding was potentiated by L-glutamate and ine with EC_{50} values of around 80 nM. In the presence of L-glutamate (10 μM), specific binding was increased 4-fold, whilst ition of glycine (10 μM) increased specific binding 2-fold. FR115427 exhibited marked stereoselectivity; (+)-FR115427 has fold higher affinity than (−)-FR115427. This ligand may therefore be useful for the pharmacological investigation of the DA receptor ion channel.

NMDA (*N*-methyl-D-aspartate); (Non-competitive), Dizocilpine; FR115427; Ligand binding

ntroduction

he *N*-methyl-D-aspartate (NMDA) receptor has n implicated in many physiological and pathological ıts in the brain. Studies using neurochemical and trophysiological techniques have revealed a num- of pharmacologically distinct modulatory sites on receptor (reviews by Reynolds and Miller, 1988;)blewski and Danysz, 1989; Wong and Kemp, 1991). bition of NMDA receptor activation can be eved via direct antagonism at the recognition site the endogenous neurotransmitter, and a number of petitive antagonists have been developed (Olver- and Watkins, 1989). Non-competitive antagonism MDA receptor mediated responses is also possible several sites; blockade of the NMDA receptor ion ınel has been investigated using drugs such as -5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclo-ten-5,10-imine (dizocilpine or MK801), the disso-ve anaesthetics phencyclidine (PCP) and ketamine, the sigma opiate, *N*-allylnormetazocine (NANM) ng et al., 1986; McDonald et al., 1987; Wong and Kemp, 1991). The glycine modulatory site of the NMDA receptor can be targetted by antagonists at this site such as 7-chlorokynurenate and L-689,560 (Kemp et al., 1988; Grimwood et al., 1991). Research aimed at developing drugs that act selectively at the polyamine recognition site of the NMDA receptor has yielded compounds such as ifenprodil and SL82.0715 (Gotti et al., 1988), and the role of tricyclic antidepressants has also been investigated (Bakker et al., 1991). Further research should reveal the role these sites play in modulating the activity of the NMDA receptor ion channel.

Radioligand binding techniques have been particularly useful in the characterisation of the NMDA receptor with the development of potent and selective ligands for the transmitter recognition site (Olverman and Watkins, 1989; Murphy et al., 1987, 1988; Sills et al., 1991), and for the ion channel (Wong et al., 1986; Bonhaus et al., 1987). [^{3}H]Dizocilpine has been widely used in radioligand binding studies to examine the NMDA receptor ion channel. This ligand binds to a single population of sites on rat brain membranes, and binding is inhibited by other NMDA receptor channel blockers such as ketamine, PCP and NANM. Studies using [^{3}H]dizocilpine have shown that binding of the

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nd is markedly enhanced by agonists for the trans-
ter recognition site, and this effect is inhibited by
ıpetitive NMDA receptor antagonists (Foster and
ng, 1987). The enhancement seems to be due to an
ease in the apparent on-rate for [^{3}H]dizocilpine
ding presumably due to the opening of the NMDA
eptor channel allowing easier access of the ligand to
binding site (Kloog et al., 1988a). When glycine is
sent a further enhancement of binding is seen, and
is probably due to the glycine-induced increase in
IDA receptor ion channel open time noted in elec-
physiological experiments (Johnson and Ascher,
7).

n the present study, we have examined the binding
racteristics of [^{3}H](+)-1-methyl-1-phenyl-1,2,3,4-te-
ıydroisoquinoline ([^{3}H]FR115427) to rat brain
mbranes. This novel dizocilpine analogue has previ-
ly been shown to interact with a site within the
IDA receptor ion channel (Hodgkiss et al., 1993).

Methods

Synthesis and radiochemical purity of [^{3}H]FR115427

^{3}H]FR115427 (18 Ci/mmol) was custom tritiated
Amersham International by catalytic reduction of a
ıo-chlorinated aromatic precursor. The stock mate-
was stored in ethanol at a concentration of 10
i/ml under liquid nitrogen. [^{3}H]FR115427 was pu-
:d on a Waters preparative resin (C-18, 55–105 μ).
ample of crude material was dissolved in eluting
ent (CH_3CN/aq. 0.1% CF_3COOH (TFA); 3:7)
applied to a 5 × 0.7 cm column of resin. The resin
eluted with 10 bed volumes of solvent and the
tions corresponding to FR115427 combined before
solvent was removed. Purified [^{3}H]FR115427 as
TFA salt was diluted to 10 μM with glass distilled
:r and stored in aliquots for experiments under
id nitrogen. Purity was confirmed by column chro-
ography as described above and by silica gel TLC
g $CHCl_3$/MeOH (9:1) as solvent. Greater than
› of the radioactivity corresponded to authentic
15427 up to 6 months after purification.

Preparation of rat brain membrane

'he cerebral cortices of male Cob-Wistar rats (250–
g) were dissected and homogenised in 15 volumes
v) of ice cold 0.32 M sucrose, using a glass teflon
ıogeniser. The homogenate was centrifuged at 1000
for 10 min at 4°C and the supernatant recen-
ged (17,000 × g, 20 min, 4°C). The resultant synap-
mal pellet was lysed with 30 volumes glass distilled
:r. After incubation at 37°C for 30 min, the mem-
ıe suspension was centrifuged at 50,000 × g for 10 min at 4°C, the supernatant discarded and the pellet washed by resuspension in glass distilled water (30 volumes) and centrifugation (50,000 × g, 10 min, 4°C). The resultant pellet was resuspended to 10 volumes and stored at −20°C. Prior to use, the 10 volume suspension was thawed and diluted to 30 volumes with glass distilled water, recentrifuged (50,000 × g, 10 min, 4°C) and the final pellet resuspended in 30 volume of 5 mM Tris-HCl (pH 7.4, 20°C) and kept on ice until required. Protein content was measured by the method of Bradford (1976).

2.3. [^{3}H]FR115427 binding assay

[^{3}H]FR115427 (10 nM) was preincubated at 25°C with 5 mM Tris-HCl buffer (pH 7.4) in the absence or presence of increasing concentrations of test drug (0.1 nM–300 μM) for 2 min prior to addition of 0.5 ml membrane suspension (30–70 μg protein). Samples were incubated at 25°C for 45 min unless otherwise indicated. L-Glutamate (10 μM) was present except when indicated. Non-specific binding was determined in the presence of 30 μM FR115427. Incubation was terminated by rapid filtration through Whatman GF/B filters presoaked in 0.05% polyethylenimine for 60 min followed by 2 × 5 ml washes using a Brandel cell harvester. Filters were transferred to scintillation vials and 100 μl formic acid (100%) was added, followed 10 min later by 4 ml Emulsifier Safe liquid scintillation fluid. Radioactivity was measured in a Canberra-Packard 1900CA liquid scintillation analyser using automatic quench correction.

2.4. [^{3}H]Dizocilpine binding assay

[^{3}H]Dizocilpine (1 nM; 24 Ci/mmol; NEN) was preincubated at 25°C with 5 mM Tris-HCl buffer (pH 7.4) in the absence or presence of increasing concentrations of test drug (0.1 nM–300 μM) for 2 min prior to addition of 0.5 ml membrane suspension (30–70 μg protein). Samples were incubated at 25°C for 120 min unless otherwise indicated. L-Glutamate (10 μM) was present except when indicated. Non-specific binding was determined in the presence of 30 μM dizocilpine. Incubation was terminated by rapid filtration through Whatman GF/B filters followed by 2 × 5 ml washes using a Brandel cell harvester and [^{3}H]dizocilpine binding was determined as described above.

3. Results

3.1. Timecourse of [^{3}H]FR115427 and [^{3}H]dizocilpine binding

Specific binding of [^{3}H]FR115427 to rat cortical synaptosomal membranes reached equilibrium within

BLE 1

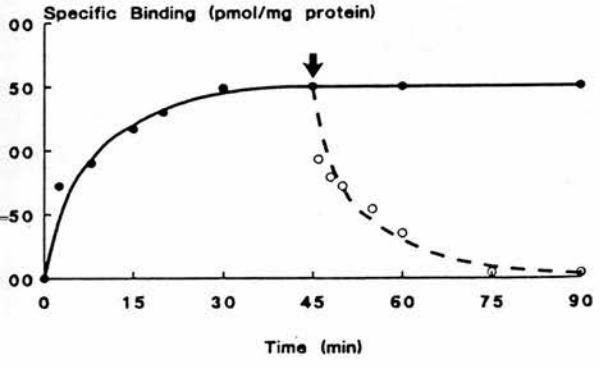
ıetic analysis of [^{3}H]FR115427 and [^{3}H]dizocilpine binding to rat cortical synaptosomal membranes

	[^{3}H]FR115427	[^{3}H]dizocilpine
$_1$ ($M^{-1}s^{-1}$)	$2.1\times10^4\pm0.32\times10^4$	$1.5\times10^5\pm0.18\times10^5$
$_1$ (s^{-1})	$9.1\times10^{-4}\pm0.62\times10^{-4}$	$2.1\times10^{-4}\pm0.25\times10^{-4}$
: K_D (nM)	45.9 ± 5.2	1.45 ± 0.22
; dissociation (min)	12.9 ± 0.9	56.6 ± 5.2

tical synaptosomal membranes were incubated with 10 nM [^{3}H]FR115427 or 1 nM [^{3}H]dizocilpine for various times at 25°C in the presence 10 μM L-glutamate. In dissociation experiments, following incubation for either 45 min ([^{3}H]FR115427) or 90 min ([^{3}H]dizocilpine), excess abelled ligand was added and binding measured at various time intervals thereafter.

min at 25°C (Fig. 1). Specific binding was reversible; : addition of 30 μM FR115427 displaced almost all :cific binding within 45 min (Fig. 1). Dissociation of ınd [^{3}H]FR115427 followed a typical single expo- ıtial decay curve, with a $t_{1/2}$ of approximately 13 ı (table 1). At equilibrium, specific binding com- sed approximately 40% of total binding at a ligand ıcentration of 10 nM.

In the same membrane preparation, [^{3}H]dizocilpine ding reached equilibrium within 120 min, and excess abelled ligand (30 μM dizocilpine) displaced almost specific binding within 150 min. The single expo- ıtial decay curve for dissociation of [^{3}H]dizocilpine d a $t_{1/2}$ of 57 min (Table 1). Specific binding at ıilibrium accounted for approximately 90% of total ınd bound at a ligand concentration of 1 nM. Ki- ic analysis of these experiments provided associa- ı and dissociation rate constants (Table 1), and gave :ulated K_D values (k_{-1}/k_{+1}) of 45.9 ± 5.2 nM for]FR115427 binding and 1.45 ± 0.22 nM for [^{3}H]di- ilpine binding.



1. Timecourse of [^{3}H]FR115427 binding to rat cortical synapto- ıl membranes. Synaptosomal membranes suspended in 5 mM HCl (pH 7.4) were incubated with 10 nM [^{3}H]FR115427 in the :nce of 10 μM L-glutamate for various times (●). Dissociation nitiated at 45 min (indicated by arrow) by the addition of 30 μM)elled FR115427, and at various times following this specific ng was measured (○). Results shown are from a single repre- ıtive experiment from a group of five independent experiments.

3.2. Effect of pH, temperature and protein concentration on [^{3}H]FR115427 binding

Specific binding of [^{3}H]FR115427 to cortical synaptosomal membranes was measured over the pH range 6.5–8.5. An optimum was apparent between pH 7.5–8.0 (Fig. 2A). Routine assays were carried out at pH 7.4 to allow direct comparison of [^{3}H]FR115427 binding with

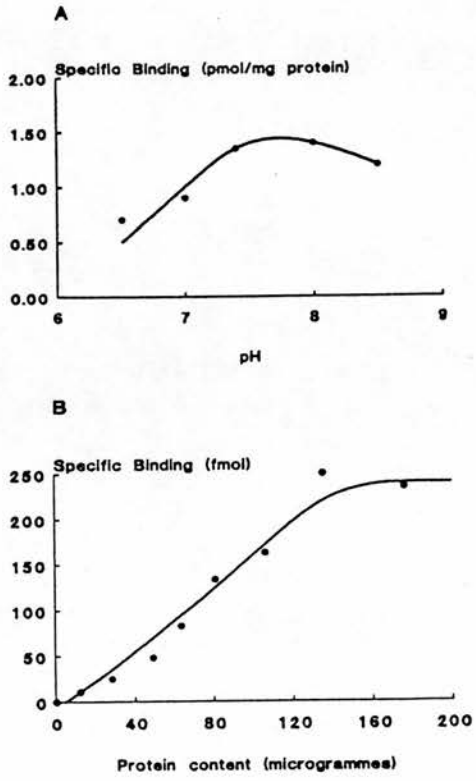
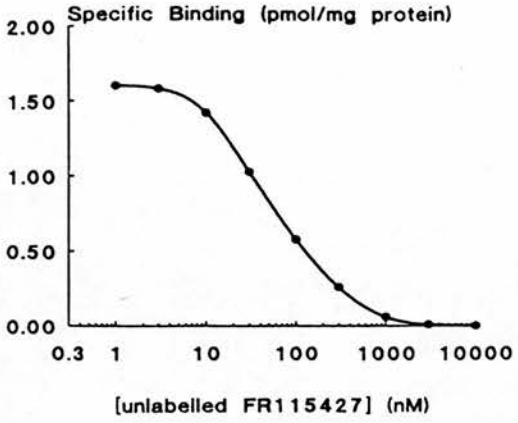


Fig. 2. (A) pH dependence of [^{3}H]FR115427 binding. Membranes were incubated in 5 mM Tris-HCl buffer at pH ranging from 6.5–8.5 with 10 nM [^{3}H]FR115427 in the presence of 10 μM L-glutamate for 45 min. Non-specific binding was defined in the presence of 30 μM FR115427. Values are mean of duplicated experiments performed in triplicate. (B) Effect of protein concentration on [^{3}H]FR115427 binding. [^{3}H]FR115427 (10 nM) was incubated with 10 μM L-glutamate for 45 min in the presence of increasing membrane protein concentration (0–170 μg/assay). Non-specific binding was defined in the presence of 30 μM FR115427. Values are from two independent experiments.



3. Inhibition of [^{3}H]FR115427 binding by unlabelled FR115427. ıbranes were incubated (45 min) with 10 nM [^{3}H]FR115427 and M L-glutamate in the absence or presence of increasing concenons of unlabelled FR115427 (1 nM–10 μM). Non-specific bindwas determined in the presence of 30 μM FR115427. Results n are from a single representative experiment from a group of independent experiments. Data from individual experiments was sed by least squares fit to the logistic expression $Y = MX^P/(X^P + IC_{50}^P)$ and K_D and B_{max} values were calculated.

]dizocilpine binding which is routinely measured at 7.4.

emperature effects were measured over the range 7°C. Specific binding remained constant with temature. Specific binding of [^{3}H]FR115427 was mead over a protein concentration range of 10–170 μg ein/tube and was linear up to 120 μg/tube (Fig. In routine experiments, a protein concentration of 70 μg/assay was used.

Determination of the K_D and B_{max} for [^{3}H]-15427 binding

he binding of [^{3}H]FR115427 was investigated by ıbating cortical synaptosomal membranes with 10 [^{3}H]FR115427 and increasing concentrations of ıbelled ligand in the presence of 10 μM L-glutae (Fig. 3). Analysis of saturation data from five ı experiments provided binding affinity (K_D) and ling site density (B_{max}) values of 45.4 ± 3.91 nM 9.12 ± 0.52 pmol/mg protein, respectively. The slope was 1.07 ± 0.15, indicating binding to a sinpopulation of sites.

Pharmacology of the [^{3}H]FR115427 binding site

he pharmacological profile of the [^{3}H]FR115427 ling site was evaluated by incubating cortical synapmal membranes with 10 nM [^{3}H]FR115427 at 25°C 45 min in the absence or presence of increasing entrations (0.1 nM–300 μM) of a number of DA receptor channel blockers (Table 2). The K_D e of (+)-FR115427 was 45.4 ± 3.91 nM, whereas

TABLE 2

Inhibition of [^{3}H]FR115427 and [^{3}H]dizocilpine binding to rat cortical synaptosomal membranes by non-competitive NMDA receptor antagonists

	K_D / K_i (nM)	
	[^{3}H]FR115427	[^{3}H]dizocilpine
(+)-FR115427	45.4 ± 3.9	35.4 ± 3.8
(−)-FR115427	4732 ± 395	3756 ± 227
(+)-Dizocilpine	6.45 ± 0.71	3.57 ± 0.4
(−)-Dizocilpine	18.4 ± 3.5	16.0 ± 1.6
Phencyclidine	35.8 ± 2.8	36.2 ± 4.5
Ketamine	576 ± 107	354 ± 59.0
N-Allylnormetazocine	304 ± 41.8	316 ± 24.8

Cortical synaptosomal membranes were incubated with 10 nM [^{3}H]FR115427 (45 min) or 1 nM [^{3}H]dizocilpine (120 min) in the presence of 10 μM L-glutamate and increasing concentrations of test drugs (0.1 nM–300 μM). Non-specific binding was determined in the presence of 30 μM unlabelled ligand. K_i values were calculated from the equation $K_i = IC_{50}/(1 + L/K_D)$. Values are mean ± S.E.M. of at least three experiments performed in duplicate.

the inhibitory constant (K_i) for (+)-dizocilpine was 6.45 ± 0.71 nM, indicating that (+)-FR115427 has a 7-fold lower affinity than (+)-dizocilpine. Binding was stereoselective since (+)-FR115427 showed 100-fold higher affinity than (−)-FR115427 (Table 2). In contrast, the affinity of (+)-dizocilpine was only 3-fold higher than its (−)-stereoisomer (Table 2). Other NMDA receptor channel blockers (PCP, ketamine, NANM) showed K_i values for displacement of [^{3}H]FR115427 binding consistent with their inhibition of [^{3}H]dizocilpine binding (Fig. 4 and Table 2). Comparison of K_i values measured against [^{3}H]FR115427 and [^{3}H]dizocilpine binding gave a regression coefficient (r) of 0.964 ($P < 0.05$; Fig. 4).

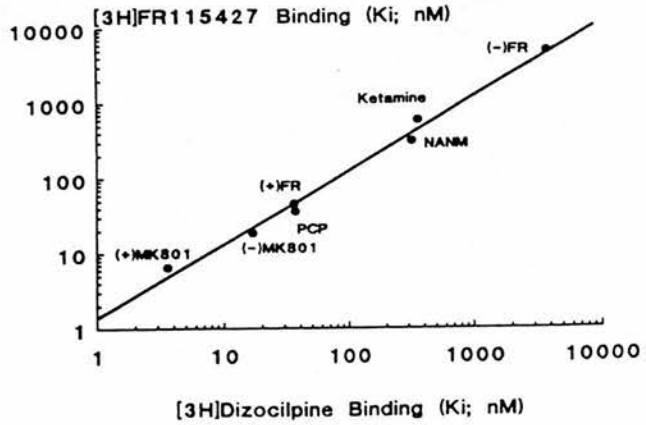
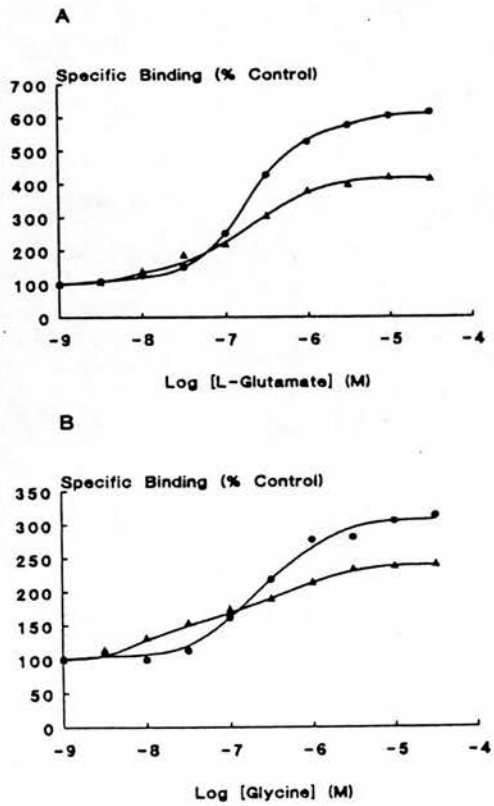


Fig. 4. Pharmacological comparison of [^{3}H]FR115427 and [^{3}H]dizocilpine binding. Membranes were incubated with 10 nM [^{3}H]FR115427 (45 min) or 1 nM [^{3}H]dizocilpine (120 min) in the presence of 10 μM L-glutamate. Correlation between the K_i values for various non-competitive NMDA receptor antagonists in the [^{3}H]FR115427 and [^{3}H]dizocilpine binding assays was evaluated by least-squares fit regression analysis ($r = 0.964$, $P < 0.05$). Non-specific binding was defined in the presence of 30 μM unlabelled ligand.

Effects of L-glutamate and glycine on [^{3}H]FR115427 [^{3}H]dizocilpine binding

In the absence of exogenous L-glutamate,]FR115427 binding was variable and specific bind- often represented less than 10% of total binding. It therefore not possible to accurately determine K_D B_{max} values for [^{3}H]FR115427 under these condi- s. L-Glutamate (10 μM) was therefore routinely ed to the assay buffer. Both L-glutamate and glycine reased specific [^{3}H]FR115427 binding to cortical aptosomal membranes, with EC_{50} values of 75.0 ± 3 nM and 93.7 ± 16.8 nM, respectively. The magni- e of the effect was different for the two transmit- ; 10 μM L-glutamate increased specific]FR115427 binding maximally to 438 ± 55.7% of trol binding (Fig. 5A), whilst 10 μM glycine in- ased specific [^{3}H]FR115427 binding maximally to ± 17.0% of control binding (Fig. 5B). In the case of cific [^{3}H]dizocilpine binding, L-glutamate (10 μM) glycine (10 μM) potentiated binding to 592 ±



. Potentiation of [^{3}H]FR115427 and [^{3}H]dizocilpine binding by tamate and glycine. Membranes were incubated with 10 nM R115427 (45 min) or 1 nM [^{3}H]dizocilpine (120 min) in the ice or presence of increasing concentrations of L-glutamate (A) cine (B). A final concentration of 1 nM–100 μM L-glutamate or glycine was added. Potentiation was calculated as a percent- of control binding in the absence of added L-glutamate or e. Data are the means (% control binding) of three experi- performed in duplicate. ([^{3}H]FR115427, filled triangle; [^{3}H]MK801, filled circle).

34.2% and 309 ± 45.0% of control binding, respectively (Fig. 5A and B). The EC_{50} values for L-glutamate and glycine enhancement of specific [^{3}H]dizocilpine binding were 194 ± 20.7 nM and 214 ± 25.6 nM, respectively.

4. Discussion

The affinity of FR115427 for the [^{3}H]]dizocilpine binding site is in the mid-nanomolar range (Hodgkiss et al., unpublished data). A ligand with a K_D of this order would be expected to have a $t_{1/2}$ for dissociation of around 15 s, a rate which would preclude the use of filtration as a method to separate bound from free ligand. However, [^{3}H]FR115427 has been shown experimentally to have the much longer $t_{1/2}$ for dissociation of 13 min. [^{3}H]FR115427 therefore behaves more like a ligand with sub-nanomolar affinity in the filtration assay, and the loss of specific binding during filtration is negligible. Theoretical calculations assuming simple binding to the receptor site would predict an association rate constant of around 10^6 M^{-1} s^{-1} (Bennett, 1978). However, kinetic analysis of [^{3}H]FR115427 binding measured in the filtration assay gave an association rate constant of 2.1×10^4 M^{-1} s^{-1}, nearly two orders of magnitude lower than the predicted value. The association rate constant for [^{3}H]dizocilpine of 1.5×10^5 M^{-1} s^{-1} was also lower than predicted, and represented a deviation from the theoretical value of 6-fold. This is consistent with published data (Kloog et al., 1988b), and in view of the kinetics of [^{3}H]FR115427 binding is probably a true reflection of the kinetics of [^{3}H]dizocilpine binding rather than an experimentally induced deviation from the predicted value. The slower association rates imply that binding of both ligands to the NMDA receptor involves more than a simple binding process, and could be related to a transition from an aqueous to a lipid environment. This proposal is consistent with data from electrophysiological studies that have suggested that the binding site for non-competitive NMDA antagonists is located in a lipophilic environment within the NMDA receptor ion channel.

Saturation analysis demonstrated that [^{3}H]FR115427 binds to a single population of sites with a B_{max} of 9.12 ± 0.52 pmol/mg protein. This binding site density is similar to the value of 8.63 ± 0.47 pmol/mg protein for [^{3}H]dizocilpine binding in the same preparation (data not shown). The K_D for [^{3}H]FR115427 binding of 45 nM was also in agreement with the K_i value of 35 nM for FR115427 measured in the [^{3}H]dizocilpine binding assay. The Hill slope was close to unity indicating a single population of binding sites. The K_i values for non-competitive NMDA receptor antagonists (PCP, ketamine, NANM) in the [^{3}H]FR115427 and [^{3}H]dizocilpine binding assays exhibit a close correlation sug-

ting that these radioligands bind to a similar site hin the NMDA receptor ion channel.

Specific binding of [^{3}H]FR115427 in the absence of igenous L-glutamate was low and variable between eriments. The EC_{50} for L-glutamate stimulation of H]FR115427 binding suggests that a low residual lutamate concentration in washed membrane prepa- ons will make a measurable difference to specific ding. Specific [^{3}H]FR115427 binding was enhanced L-glutamate and this amino acid was therefore rou- ly added to the binding assay. The presence of a ırating concentration of L-glutamate (10 μM) in- ased specific binding 4-fold. A 2-fold increase in cific [^{3}H]FR115427 binding was seen in the pres- e of 10 μM glycine. In contrast, [^{3}H]dizocilpine ding was measurable in the absence of added exoge- ıs L-glutamate due to its higher affinity for the HDA receptor ion channel. The effects of L-gluta- e and glycine on [^{3}H]dizocilpine binding and]FR115427 binding also showed differences; the $_{50}$ values for stimulation of [^{3}H]FR115427 binding -glutamate and glycine were approximately 80 nM, reas for [^{3}H]dizocilpine binding the EC_{50} values e 2–4-fold higher. The degree of potentiation was, ever, similar with specific [^{3}H]dizocilpine binding ıg increased 6-fold and 3-fold by L-glutamate (10) and glycine (10 μM), respectively.

Although the affinity of [^{3}H]FR115427 for the DA receptor ion channel is approximately 10-fold er than that of [^{3}H]dizocilpine, thereby making it a attractive radioligand for the study of this site, the eoselectivity of FR115427 is much greater. The -isomer of FR115427 is approximately 100-fold e potent than the corresponding (−)-isomer, whilst e is only a 3-fold separation in the affinities of the eoisomers of dizocilpine (the (+)-isomer having higher affinity). [^{3}H]FR115427 may therefore be a ul pharmacological tool for the study of the NMDA ptor ion channel.

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